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PART I

The Journal of Clinical Investigation

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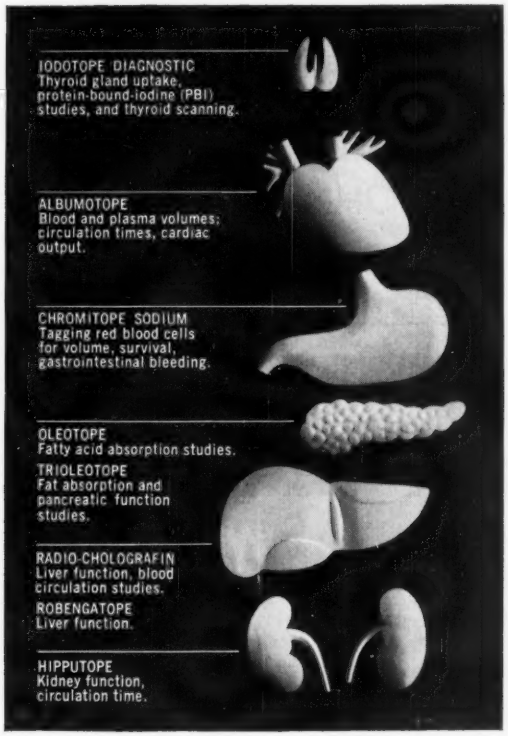

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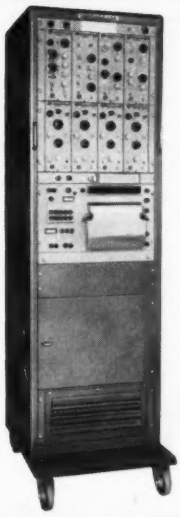

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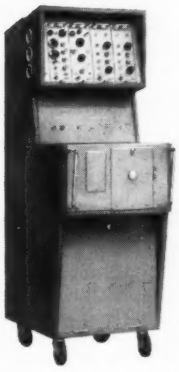

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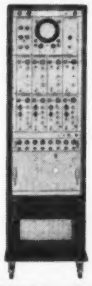

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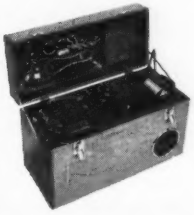

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(1) Matthias, J. Q.; Misiewicz, J. J., and Scott, R. B.: Brit. M. J. *2*:1837-1840 (Dec. 24) 1960.
(2) Coggins, P. R.; Ravdin, R. G., and Eisman, S. H.: Cancer *13*:1254-1260 (Nov.-Dec.) 1960.
(3) Papac, R.; Petrakis, N. L.; Amini, F., and Wood, D. A.: J.A.M.A. *172*:1387-1391 (March 26) 1960.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* —Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2-3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan—Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage—The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2-3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

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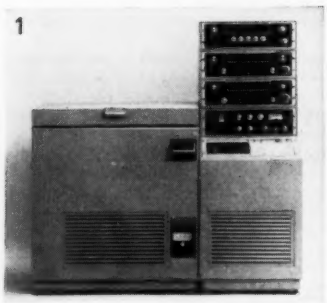

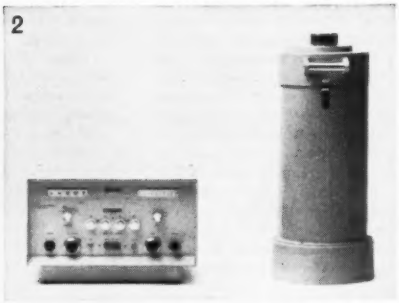

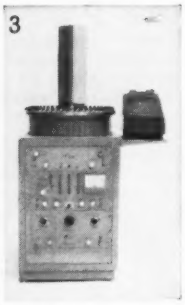

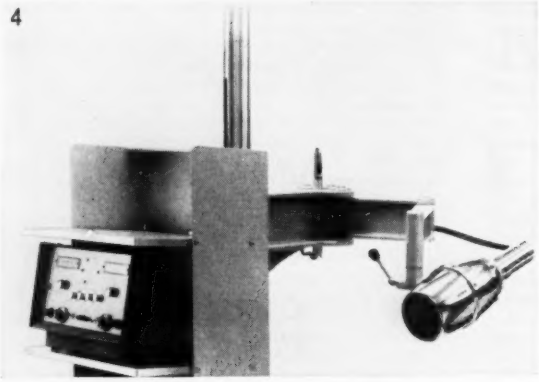

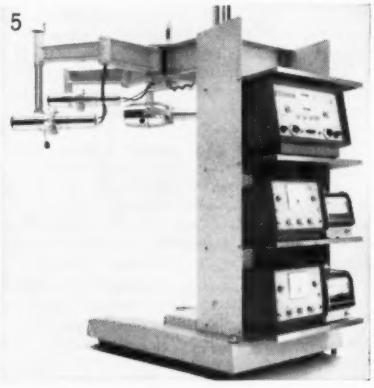

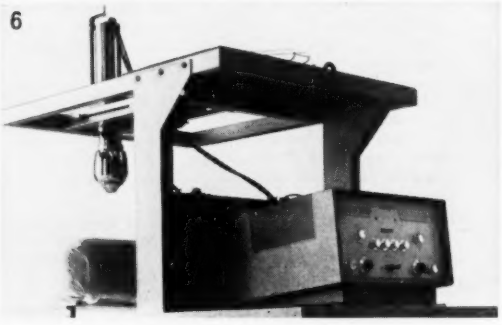

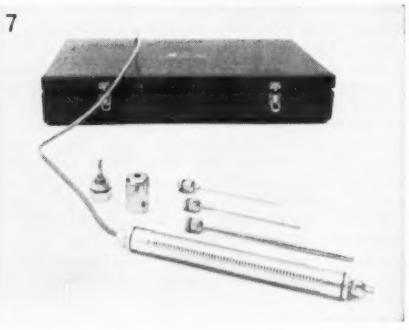

1. Automatic, temperature-controlled liquid scintillation counting system Model 703 shown with two scalers, timers and digital recorder. For counting large numbers of carbon-14 and tritium samples with highest efficiency and precision.

2. Scintillation well detector Model DS-303 and spectrometer Model 132B for measurement of radioactivity in urine, blood and plasma.

3. Automatic system for analysis of gamma samples employs Model C120 sample changer with shielded well scintillation detector plus printing timer, scaler and spectrometer.

4. Probe scintillation detector with three-inch crystal and scaler-spectrometer shown mounted on Model 1710 clinical stand constitute instrumentation for thyroid uptake measurements.

5. Versatile system for in vivo procedures. Clinical stand supports as many as three scintillation detectors together with associated equipment such as ratemeters, recorders, and a scaler-spectrometer for evaluation of thyroid and kidney function and estimation of cardiac output and myocardial blood flow.

6. Model 1700A isotope scanner produces graphic record of radioisotope distribution in body area 14 x 17 inches. Used with collimated scintillation detector.

7. Model DS8-1 light-weight needle-probe scintillation detector set is furnished with four probes. Used with scalers, ratemeters or gamma spectrometer systems to localize radioisotope concentrations in tissue during surgery.

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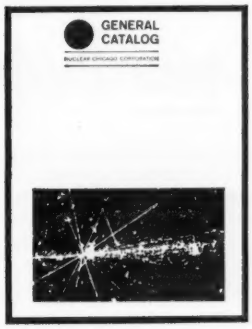

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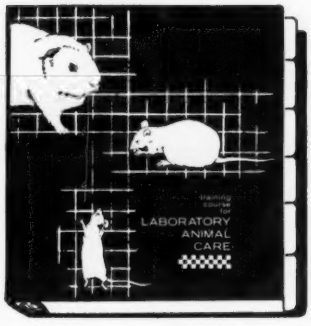

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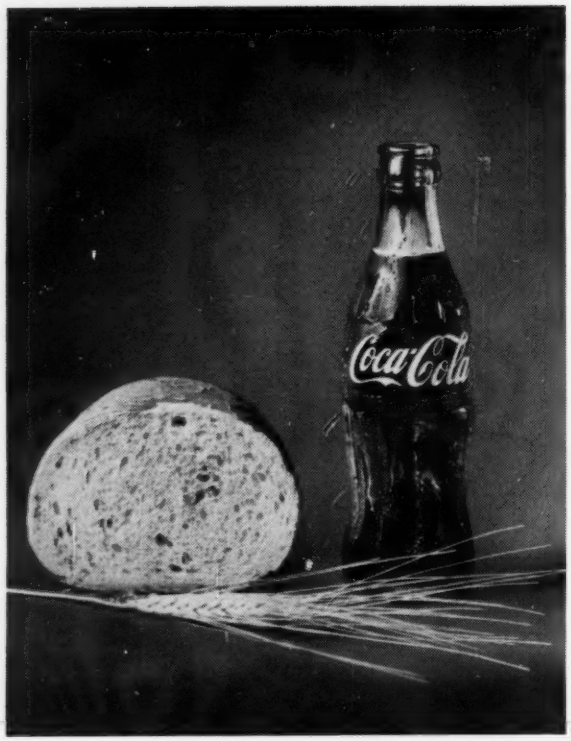

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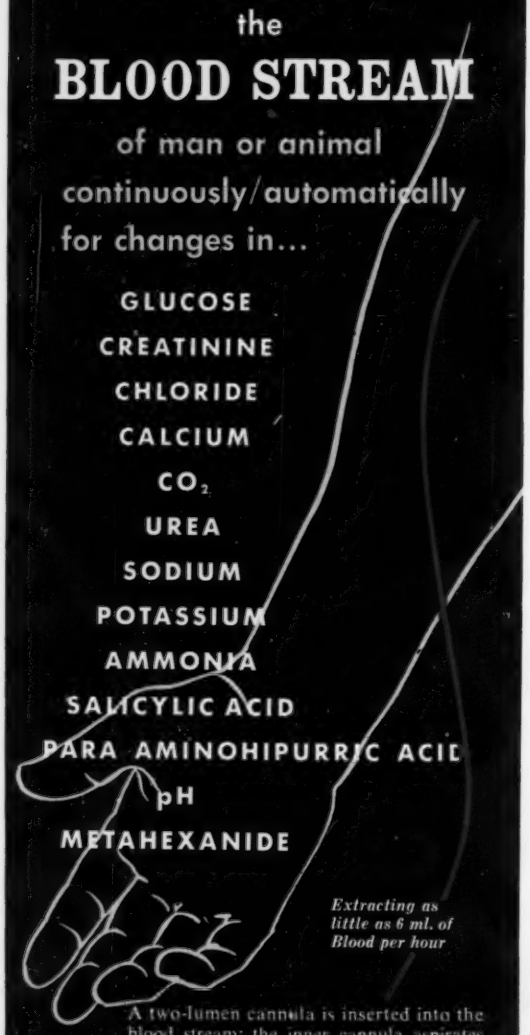

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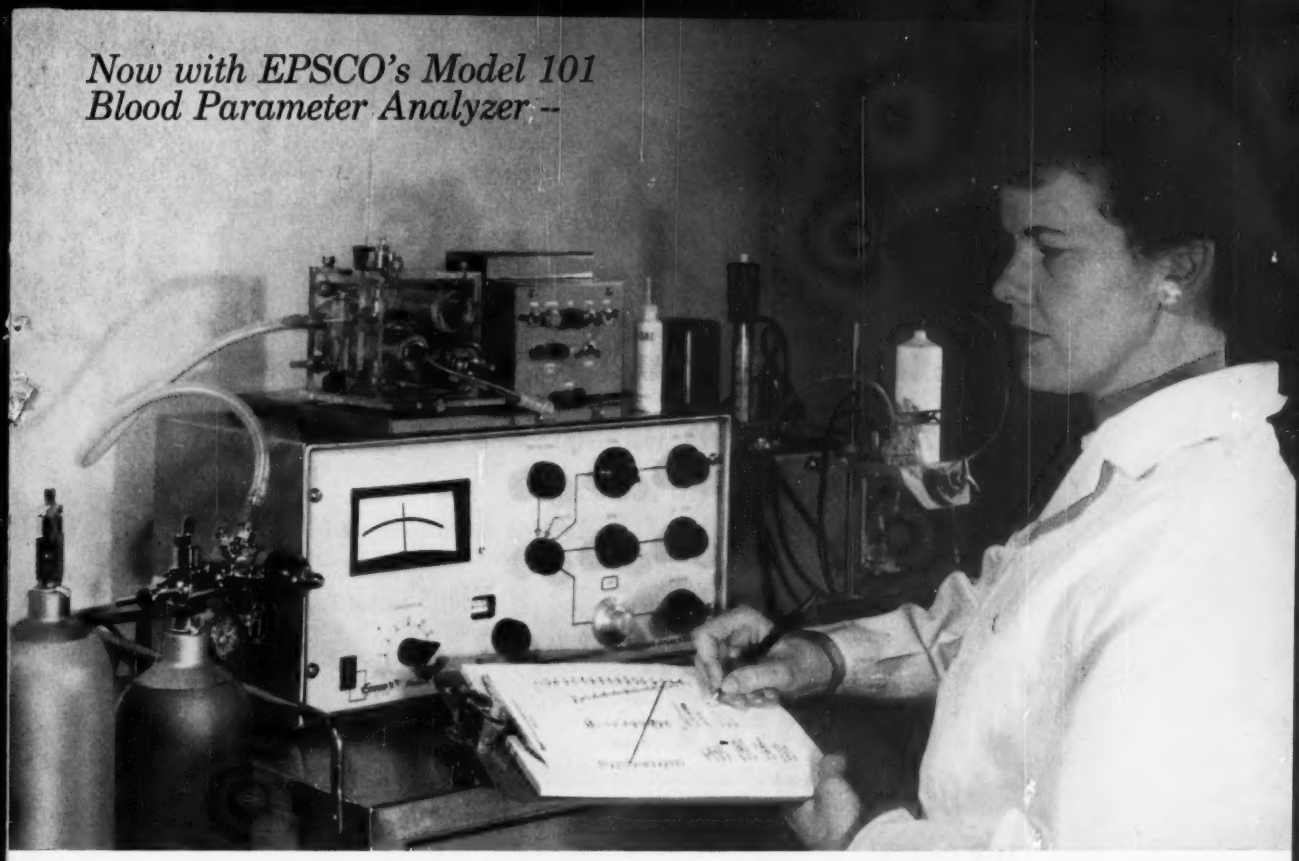

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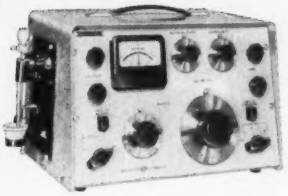

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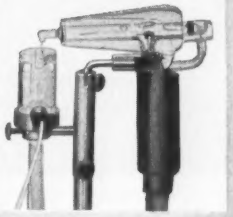

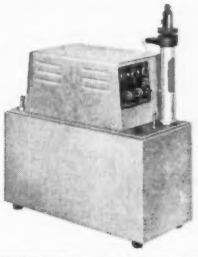

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STUDIES ON THE PATHOGENESIS OF THE ETHANOL-INDUCED FATTY LIVER. I. SYNTHESIS AND OXIDATION OF FATTY ACIDS BY THE LIVER *

By GILBERTO REBOUÇAS † AND KURT J. ISSELBACHER

(From the Department of Medicine, Harvard Medical School and the Gastroenterology Unit of the Medical Services, Massachusetts General Hospital, Boston, Mass.)

(Submitted for publication November 28, 1960; accepted March 17, 1961.

In studying the effects of ethanol on the liver, we have directed our attention to the manner by which ethanol intoxication in animals causes an acute fatty liver (1, 2). Although the mechanism involved is still not well understood, the production of the fatty liver under these conditions probably involves one or more of the following four processes: *1*) increased hepatic lipid synthesis, *2*) decreased hepatic lipid utilization (e.g., oxidation), *3*) increased transport of fatty acids from peripheral fat depots or *4*) decreased release of lipid from the liver. Mallov and Bloch (1) originally suggested that the fatty liver following ethanol administration was due to an increased transport of fat from the depots to the liver, but later (3) considered that the increased hepatic lipid might be the result of increased hepatic fatty acid synthesis. Consistent with the latter concept are the observations of Lieber, DeCarli and Schmid (4, 5) that ethanol, *in vivo* and *in vitro*, stimulates fatty acid synthesis in the liver. These authors attributed the enhanced fatty acid synthesis to an increased production of reduced diphosphopyridine nucleotide (DPNH) produced by the oxidation of ethanol (5). On the other hand, good evidence has been presented recently (6, 7) that increased transport of fatty acids to the liver from the fat depots may be the major mechanism for the fatty liver produced by ethanol as well as by other agents, such as ethionine and carbon tetrachloride.

To gain further insight into the mechanism whereby ethanol intoxication produces a fatty liver, we have investigated the four processes referred to above in order to determine the extent to which one or more of them may be involved. The present report is a study of changes in hepatic fatty acid synthesis and oxidation following ethanol administration *in vivo* and *in vitro*. Observations on the role of reduced and oxidized diphosphopyridine nucleotides are also presented. A subsequent report will deal with studies on lipid transport.

MATERIALS AND METHODS

Palmitate-1-C^{14} (specific activity 5×10^6 cpm per μmole) was obtained from Volk Radiochemical Co., Chicago, Ill., and purified as described previously (8). Acetate-1-C^{14} was purchased from New England Nuclear Corporation, Boston, Mass. Diphosphopyridine nucleotide (DPN) was obtained from Sigma Chemical Co., St. Louis, Mo. Hyamine-10X was prepared according to the method of Passman, as modified by Eisenberg (9). Female albino rats (Charles River Laboratories, Boston, Mass.), weighing between 180 and 250 g and maintained on Purina Chow were used throughout this investigation.

Acetate-1-C^{14} incorporation into liver lipid. The animals were given, by stomach tube, 9.3 g per kg ethanol (50 per cent by volume), isocaloric amounts of glucose or sorbitol, or equivalent volumes of saline and then fasted for 16 hours. At the end of this period 1 ml of saline containing 20 μc acetate-1-C^{14} (specific activity 25 to 27 μc per mg) was injected into the portal vein with the animals under light ether anesthesia. The rats were sacrificed 45 minutes later. When the animals were pretreated with nicotinamide, they received 500 mg per kg i.p. daily for 3 days prior to ethanol administration. Control animals were injected with saline. Studies on hypophysectomized rats (Charles River Laboratories) were not carried out until 2 weeks after the operation. In the experiments on cordotomized animals, the operation was performed at the C-4 to C-6 level 1 to 4 hours prior to ethanol or saline treatment. Both hypophysectomized and cordotomized rats, because of a greater sensitivity to ethanol intoxication, received a smaller dose of ethanol (6.2 g per kg).

At the end of the *in vivo* part of the experiment, the animals were killed by cervical dislocation and the livers quickly excised. Lipid was extracted with chloroform-methanol according to Folch, Lees and Sloane Stanley (10). The chloroform fraction was evaporated to dryness and taken up in 10 ml petroleum ether. Aliquots were assayed for radioactivity in a Packard Tri-Carb

* This work has been supported in part by Grant A-1392 from the National Institutes of Health, Bethesda, Md.

† Fellow of the W. K. Kellogg Foundation and the American College of Physicians. Present address: Hospital das Clinicas, Salvador, Bahia, Brazil.

TABLE I

Some factors influencing lipid accumulation and C^{14}-acetate incorporation into lipids in rat liver

No. of animals	Treatment*	Total liver fatty acids†	C^{14}-acetate incorporated into total liver lipid
		mg/g	*% injected cpm/g liver*
6	Saline	36.57 ± 2.13	0.058 ± 0.016
6	Glucose	32.42 ± 1.99 (p 0.001)‡	0.250 ± 0.113 (p 0.001)
6	Ethanol	71.58 ± 13.20 ($p < 0.001$)	0.379 ± 0.264 ($0.001 < p < 0.005$)
7	Sorbitol	38.77 ± 6.25 ($p > 0.1$)	0.233 ± 0.128 (p 0.001)

* Rats were intubated with ethanol (9.3 g/kg), isocaloric amounts of glucose or sorbitol, or equivalent volumes of saline and then fasted for 16 hours. Twenty μc C^{14}-acetate was injected into the portal vein and the animals sacrificed 45 minutes later.

† Mean ±SD.

‡ p Values compared with saline group as control.

liquid scintillation spectrometer, as described previously (8), and analyzed for total fatty acids by the method of Albrink (11).

Palmitate-1-C^{14} oxidation to $C^{14}O_2$

I. Liver homogenate studies. Rats were either fasted for 16 hours prior to the experiments or given ethanol, glucose or saline (in the doses mentioned above) and then sacrificed in 16 hours. When the animals were pretreated with nicotinamide, they received 500 mg per kg i.p. daily for 3 days. At the conclusion of the *in vivo* portion of the experiment, the animals were sacrificed, the livers excised and 1:1 (weight: volume) homogenates prepared in 0.15 M potassium chloride containing 0.05 M nicotinamide. The homogenization was performed at 0 to 4°C using a Dounce homogenizer. The standard incubation system consisted of 50 mμmoles palmitate-1-C^{14}, 80 μmoles potassium phosphate buffer at pH 7.4, and approximately 15 mg liver protein in a final volume of 1.5 ml. The incubations were carried out in 25-ml Erlenmeyer flasks provided with a center well. The flasks were gassed with 95 per cent oxygen-5 per cent CO_2, then sealed with rubber serum caps and agitated for 30 minutes at 37°C in a Dubnoff shaking incubator. The reaction was stopped by the injection of 0.25 ml 10 N sulfuric acid into the outer well as described previously (12). One ml Hyamine was then injected into the center well and the flasks agitated for an additional 30 minutes to trap the $C^{14}O_2$. Aliquots of the Hyamine-$C^{14}O_2$ were assayed for radioactivity in the liquid scintillation spectrometer (12).

II. In vivo studies. Rats were given ethanol, glucose or saline and after 4 or 16 hours were injected with 250 mμmoles palmitate-1-C^{14} i.p. They were then placed in special respiratory cages and the expired CO_2 trapped in Hyamine as described by Fredrickson and Ono (13). $C^{14}O_2$ was counted as described above and the $C^{12}O_2$ determined by titration with 0.1 N hydrochloric acid.

DPN and DPNH levels in rat liver. The rats were killed by cervical dislocation and the livers irrigated

TABLE II

DPN and DPNH levels in rat liver

Treatment	No. of animals	DPN	DPNH	DPN+DPNH	Ratio DPN/DPNH
			*mμmoles/g liver protein**		
Saline†	14	2,237 ± 271	384 ± 122	2,636 ± 316	6.32 ± 1.90
Ethanol†‡	8	2,070 ± 553	696 ± 251	2,767 ± 793	3.12 ± 0.25
Sorbitol‡	7	2,269 ± 606	561 ± 213	2,831 ± 228	4.37 ± 1.52
Nicotinamide§	7	3,669 ± 848	645 ± 309	4,314 ± 928	5.65 ± 0.82
Nicotinamide§ plus ethanol‡	7	4,231 ± 1,884	916 ± 397	5,147 ± 2,074	5.01 ± 2.33
Cordotomy	4	2,256 ± 310	526 ± 32	2,782 ± 334	4.52 ± 0.63
Cordotomy plus ethanol‖	4	1,965 ± 201	760 ± 178	2,725 ± 106	2.66 ± 0.45

* Mean ±SD.

† Data from Reference 16.

‡ Ethanol, 9.3 g/kg, by stomach tube 16 hours previously; sorbitol in isocaloric amounts (16.3 g/kg).

§ Nicotinamide, 500 mg/kg i.p. daily for 3 days.

‖ Ethanol, 6.2 g/kg, by stomach tube 1 to 2 hours after cordotomy and 16 hours before sacrifice.

with 3.0 ml of 0.15 M potassium chloride containing 0.05 M nicotinamide. The livers were blotted and weighed and then homogenized with 15 ml of cold 0.25 M sucrose -0.05 M nicotinamide in a chilled metal Waring blender. One-ml aliquots were removed and homogenized with 2.0 ml of 10 per cent trichloroacetic acid for DPN measurements. For DPNH determinations, 2-ml aliquots of the homogenate were added to 3 ml of boiling sodium carbonate (0.167 M). After boiling for 3 to 5 minutes the mixtures were rehomogenized for 30 seconds and placed on ice. DPN and DPNH were then measured fluorometrically by the methyl ethyl ketone method of Ciotti and Kaplan (14). Protein was measured by the biuret reaction (15).

RESULTS

Incorporation of acetate-1-C¹⁴ into hepatic lipid

Sixteen hours after the administration of 9.3 g per kg of ethanol to female rats, there was a twofold increase in the total fatty acid content of the liver which occurred together with an increased incorporation of labeled acetate into liver lipid (Table I). It was found that ethanol and other agents, such as sorbitol, also produce a significant increase in the hepatic levels of DPNH (Table II). This increase in DPNH is a reflection of the oxidative metabolism of these substances in the liver by the DPN-linked dehydrogenases, alcohol dehydrogenase and sorbitol dehydrogenase. It was of interest that sorbitol, like ethanol, stimulated the incorporation of C^{14}-acetate into hepatic lipid (Table I). However, in contrast to ethanol, treatment of rats with sorbitol did not produce an increase in liver fat. When rats were given glucose in amounts isocaloric to ethanol, there was also a significant stimulation of the incorporation of labeled acetate into lipid, but the liver lipid content was actually decreased under these conditions (Table I).

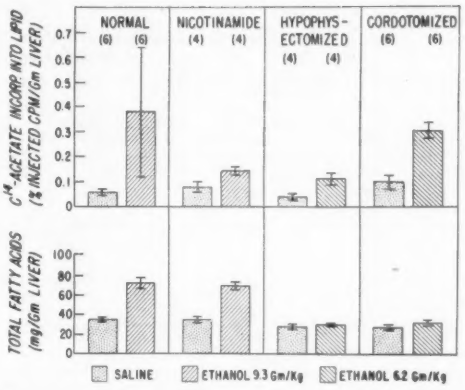

FIG. 1. C^{14}-ACETATE INCORPORATION INTO LIPID AND THE AMOUNT OF HEPATIC LIPID FOLLOWING VARIOUS EXPERIMENTAL PROCEDURES. For details see text. Numbers in parentheses refer to the number of animals.

Further evidence for the dissociation between acetate-1-C^{14} incorporation into lipid and the development or presence of a fatty liver is provided in Figure 1. Rats were given nicotinamide (500 mg per kg) for 3 days prior to ethanol treatment in order to increase their hepatic levels of DPN and DPNH (Table I) (17). It is evident that nicotinamide pretreatment greatly reduced the stimulatory effect of ethanol on C^{14}-acetate incorporation into lipid but did not reduce the extent of the fatty liver. Studies were also carried out in hypophysectomized and cordotomized animals. These experiments were based on the observations of Mallov and Bloch (1) that hypophysectomy prevents the development of a fatty liver following acute ethanol intoxication in rats, and of Calvert and Brody (7) that cordotomy abolishes the toxic effects of carbon tetrachloride on the liver. It will be seen in Figure 1 that in

TABLE III

*Effect of in vitro addition of ethanol, sorbitol or xylitol on C^{14}-palmitate oxidation in rat liver homogenates**

No. of animals	Addition	Final conc.	C^{14}-palmitate recovered as $C^{14}O_2$†		Change
			Without addition	With addition	
		moles	*mμmoles/100 mg protein*		%
8	Ethanol	0.570	21	11	−48
2	Sorbitol	0.013	23	17	−26
2	Xylitol	0.013	24	19	−21

* All animals were fasted for 16 hours before sacrifice. Each incubation flask contained: 50 mμmoles C^{14}-palmitate, 1 ml liver homogenate containing approximately 15 mg protein, plus the addition. Total volume 1.5 ml.

† Figures represent the mean values of all experiments.

TABLE IV

Effect of in vivo ethanol administration on C^{14}-palmitate oxidation in rat liver homogenates and the influence of nicotinamide pretreatment

Group	No. of animals	Substance administered*	Nicotinamide pretreatment†	C^{14}-palmitate recovered as $C^{14}O_2$‡	Change§
				mμmoles/100 mg protein	%
1	14	Saline	0	16 ± 4	
2	3	Saline	+	28 ± 3 ($p < 0.001$)§	+75
3	14	Ethanol	0	11 ± 3 ($p < 0.001$)	−31
4	4	Ethanol	+	25 ± 6 ($p < 0.005$)	+62

* Ethanol, 9.3 g/kg, as a 50% solution by volume or equivalent volumes of saline by stomach tube 16 hours before sacrifice.
† Nicotinamide, 500 mg/kg, i.p. daily for 3 days prior to ethanol or saline administration.
‡ Mean ±SD.
§ Compared with group 1 as control.

the hypophysectomized and cordotomized animals, ethanol failed to produce an increase in the total liver fat but under these conditions there was still a definite stimulation of C^{14}-acetate incorporation into lipid. It should be noted that these animals, because of their reduced ethanol tolerance, received a smaller dose (6.2 g per kg). This amount of ethanol, however, is quite effective in producing a fatty liver in normal rats (1).

Palmitate-1-C^{14} oxidation to $C^{14}O_2$

In vitro studies. The addition of ethanol to homogenates of normal rat liver consistently produced a depression in the oxidation of C^{14}-palmitate to $C^{14}O_2$. In Table III it will be noted that at a final ethanol concentration of 0.57 M there was a mean inhibition of 48 per cent. Since it seemed possible that this effect might be secondary to increased levels of DPNH, as had been shown to be the case for the impaired galactose oxidation produced by ethanol (12, 18), palmitate oxidation was measured in the presence of sorbitol and xylitol, substances which also serve to generate DPNH. Sorbitol and xylitol addition to the homogenates led to a similar although less pronounced inhibition as compared with ethanol (Table III). It should be noted, however, that the final concentration of these sugars in the homogenates was considerably below that of ethanol.

When palmitate oxidation was measured in liver homogenates of animals given ethanol (9.3 g per kg) 16 hours previously, the oxidation was 31 per cent below that observed in homogenates from the livers of animals intubated with saline (Table IV). Evidence was obtained that this inhibition was not simply a nonspecific toxic effect of ethanol. Table IV shows that pretreatment of the rats with nicotinamide entirely reversed the inhibitory effect of ethanol on the oxidation of C^{14}-palmitate to $C^{14}O_2$. In fact, in these homogenates, palmitate oxidation was 62 per cent greater than in those prepared from animals intubated with saline and not pretreated with nicotinamide.

Reversal of the inhibition of palmitate oxidation to CO_2 was also readily obtained simply by increasing the DPN concentration in the homogenates. Table V shows that the addition of DPN (2.75×10^{-3} M) to the incubation medium significantly increased the rate of palmitate oxidation in normal liver homogenates (+ 170 per cent)

TABLE V

Effect of DPN on C^{14}-palmitate oxidation in liver homogenates of rats given saline or ethanol

No. of animals	Substance administered*	C^{14}-palmitate recovered as $C^{14}O_2$		Change due to DPN addition
		Without DPN	With DPN†	
		mμmoles/100 mg protein		%
4	Saline	17	46	+170
4	Ethanol	12	22	+83

* Ethanol, 9.3 g/kg, as a 50% solution by volume, or equivalent volume of saline by stomach tube 16 hours prior to sacrifice.
† DPN, 2.75×10^{-3} M, added to the incubation flask.

as well as in homogenates of ethanol-treated animals (+ 83 per cent).

In vivo studies. Experiments were carried out to measure the respiratory $C^{14}O_2$ production following the intraperitoneal injection of palmitate-1-C^{14} in control animals (saline-treated) versus those receiving ethanol 16 hours previously. These results, shown in Figure 2, reveal a decrease in the labeling of the respiratory CO_2 in the ethanol-treated animals compared with the saline controls. Similar results were obtained when the palmitate was injected 4 hours after ethanol or saline. It is of interest that when glucose was given in amounts isocaloric to ethanol, there was a depression in the liberation of respiratory $C^{14}O_2$ which was even greater than that observed in the ethanol-treated animals. However, this depression of C^{14}-palmitate oxidation by glucose, which has also been observed by Lossow and Chaikoff (19), does not result in an increase in total liver lipid.

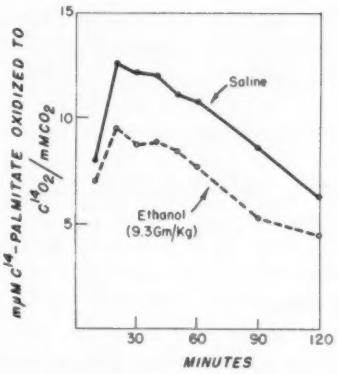

FIG. 2. EFFECT OF ETHANOL ADMINISTRATION ON PALMITATE-1-C^{14} OXIDATION TO $C^{14}O_2$ *IN VIVO*. The animals were intubated with saline or ethanol 16 hours before receiving 250 mμmoles of palmitate-1-C^{14} intraperitoneally. Respiratory $C^{12}O_2$ and $C^{14}O_2$ were measured as described under Methods. The points are mean values obtained from 4 animals in each group.

Alterations in hepatic levels of DPN and DPNH

In Table II are recorded the changes in hepatic levels of DPN and DPNH following the administration of saline, ethanol, sorbitol, nicotinamide and nicotinamide plus ethanol. The effects of cordotomy, with and without subsequent ethanol treatment, are also recorded. As referred to above, ethanol at a dose of 9.3 g per kg produced almost a doubling of the DPNH levels in the liver without changing appreciably the total amount of DPN plus DPNH. As a result, the DPN/DPNH ratio in the liver tissue of these animals dropped from 6.32 ± 1.90 (saline controls) to 3.12 ± 0.25. Comparable results have been reported by Smith and Newman (20) in rats receiving smaller doses of ethanol (3 g per kg). The administration of isocaloric amounts of sorbitol produced a similar but less striking drop in the DPN/DPNH ratio. Nicotinamide treatment produced not only a significant rise in DPN levels as described by Kaplan and co-workers (17) but also a pronounced increase in DPNH. As a result, the DPN/DPNH ratio was 5.65 ± 0.82. Of note was the fact that when the nicotinamide-injected rats were given ethanol, there was a still further increase in both DPN and DPNH resulting in a DPN/DPNH ratio of 5.01 ± 2.33. This nicotinamide pretreatment prevented the marked drop in the DPN/DPNH ratio caused by ethanol alone.

The cordotomized rats were found to have somewhat higher levels of DPNH than the saline controls and as a result the mean DPN/DPNH ratio was also lower (4.52 ± 0.63). However, when the cordotomized rats received ethanol, even though it was at a lower dose (6.2 g per kg), there was still a significant drop in the DPN/DPNH ratio to 2.66 ± 0.45.

DISCUSSION

From the foregoing experiments and those reported by Lieber and co-workers (4, 5) it is evident that ethanol administration to rats stimulates the hepatic synthesis of fatty acids. This increased fatty acid synthesis, as reflected by the enhanced incorporation of C^{14}-acetate into lipid, appears to be related to the increased amounts of DPNH generated by the metabolism of ethanol in the liver. However, from the additional experiments with sorbitol and nicotinamide, it would appear that the incorporation of C^{14}-acetate is more closely related to the DPN/DPNH ratio than to the actual levels of DPNH in the liver cells.

It should be emphasized that reduced triphosphopyridine nucleotide (TPNH) is perhaps more important and rate-limiting than is DPNH in the synthesis of fatty acids (21–23) by the liver. In

fact, in the "malonate pathway" of fatty acid synthesis, believed by Green and Wakil (24) to be the major route for the *de novo* synthesis of long chain fatty acids, TPNH, not DPNH, is the required hydrogen donor. However, fatty acid synthesis also takes place in mitochondria by reversal of fatty acid oxidation and, in this scheme, DPNH is an essential cofactor (25, 26).

Although it might be postulated that the fatty liver induced by ethanol is primarily the result of increased hepatic fatty acid synthesis, our experiments do not support such a concept. It was noted that agents such as sorbitol or glucose, when given in amounts isocaloric to ethanol, were quite effective in stimulating C^{14}-acetate incorporation into lipid but neither produced an increase in liver fat. In fact, in the glucose-treated animals the total liver lipid was actually decreased.

Of importance was the observation that pretreatment of rats with nicotinamide significantly diminished the incorporation of C^{14}-acetate into lipid and yet the extent of the fatty liver was the same as in animals receiving ethanol alone (Figure 1). On the other hand, hypophysectomized and cordotomized animals failed to develop a fatty liver following ethanol administration, but the C^{14}-acetate incorporation into lipid was still increased, especially in the cordotomized rats (Figure 1).

The dissociation of C^{14}-acetate incorporation and the accumulation of fat in the liver demonstrated in these experiments, makes it unlikely that increased fatty acid synthesis by the liver is a major or important factor in the fatty liver induced by ethanol. It remains to be determined whether hepatic fatty acid synthesis is also unimportant in other forms of experimentally induced fatty liver.

Theoretically, an impairment in the utilization or oxidation of fatty acids by the liver might also be responsible for an increase in hepatic lipid. In this connection it was observed that the oxidation of palmitate-1-C^{14} to $C^{14}O_2$ in the whole animal was inhibited by ethanol administration. However, the administration of isocaloric amounts of glucose to rats produced a depression of palmitate oxidation *in vivo* which was even greater than that observed with ethanol; yet there was no increase in liver fat in the glucose-fed rats.

In studies on rat liver homogenates, the addition of ethanol to the incubation system caused a decrease in the production of $C^{14}O_2$ from C^{14}-palmitate which seemed related, at least in part, to changes in the state of the oxidized and reduced pyridine nucleotides produced by ethanol. Thus, addition to the homogenates of two other substances which generate DPNH (i.e., sorbitol and xylitol), also caused a depression in $C^{14}O_2$ formation. On the other hand, addition of DPN to the incubation system, or the use of liver homogenates from nicotinamide-injected rats, completely prevented the inhibitory effect of ethanol on C^{14}-palmitate oxidation to $C^{14}O_2$. Lieber and Schmid (5) have also observed an inhibition by ethanol of palmitate-1-C^{14} oxidation to $C^{14}O_2$ in liver slices. They have suggested that this inhibition may be due to a decrease in the oxidation of tricarboxylic acid cycle intermediates by DPNH.

It should be emphasized that any interpretations of the changes in the conversion of palmitate-1-C^{14} to $C^{14}O_2$ *in vivo* and *in vitro* are complicated by possible alterations in pool size. In the *in vivo* experiments the mobilization of free fatty acids from adipose tissue by ethanol (6, 27) will tend to dilute the C^{14}-palmitate pool. At the same time the effects of fasting will tend to increase palmitate oxidation (28). The acetyl coenzyme A pool from which the $C^{14}O_2$ is derived will also tend to be diluted by the oxidative metabolism of ethanol to acetate and acetyl coenzyme A. Nevertheless, it is evident from the experiments with nicotinamide that the effect of ethanol on palmitate oxidation can be dissociated from the fatty liver induced by ethanol.

The experiments presented above do not provide the definitive answer as to the mechanism involved in the development of the ethanol-induced fatty liver. They serve to emphasize, however, that increased hepatic fatty acid synthesis and impaired fatty acid oxidation by the liver are not major or primary factors in this process. The inference is drawn that derangements in lipid transport are probably the significant factors in the pathogenesis of the fatty liver. In a recent preliminary report Maling and associates (6) suggest that ethanol and agents such as ethionine and carbon tetrachloride induce a fatty liver by in-

creasing fat mobilization from peripheral adipose tissues. However, Recknagel, Lombardi and Schotz (29), on the basis of experiments with carbon tetrachloride, favor the concept that the fatty liver results primarily from a decrease in the release of lipid from the liver. In view of these conflicting theories, further experiments are in progress in this laboratory to evaluate the role of alterations in lipid transport in the pathogenesis of the fatty liver produced by ethanol.

SUMMARY

1. In order to elucidate the mechanism involved in the fatty liver induced by ethanol intoxication in rats, studies have been carried out on the effects of ethanol on the incorporation of acetate-1-C^{14} into lipid and the oxidation of palmitate-1-C^{14} to $C^{14}O_2$ in liver.

2. Ethanol administration to rats stimulated the incorporation of acetate-1-C^{14} into liver lipids. However, glucose and sorbitol stimulated C^{14}-acetate incorporation into lipid without producing an increase in liver fat.

3. Injection of animals with nicotinamide prior to ethanol treatment depressed the incorporation of C^{14}-acetate into lipid, but the extent of the fatty liver was unaltered. Conversely, hypophysectomy and cordotomy prevented the fatty liver, but C^{14}-acetate incorporation remained increased.

4. Ethanol produced almost a doubling in the amount of reduced diphosphopyridine nucleotide (DPNH) in the liver and a decrease in the DPN/DPNH ratio. However, increasing the DPNH levels or decreasing the DPN/DPNH ratio by other means, such as by sorbitol administration, did not result in a fatty liver.

5. Palmitate-1-C^{14} oxidation to $C^{14}O_2$ was depressed by ethanol, both *in vivo* and *in vitro*. Prior treatment of the animals with nicotinamide completely prevented this inhibitory effect of ethanol but the extent of the fatty liver was unchanged.

6. It is concluded that the fatty liver following acute ethanol intoxication in rats is not directly related to an increased synthesis or a decreased oxidation of fatty acids in the liver. Derangements in lipid transport are probably the important factors in the production of the ethanol-induced fatty liver.

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THE AMINO ACID CONTENT OF HUMAN CEREBROSPINAL FLUID IN NORMAL INDIVIDUALS AND IN MENTAL DEFECTIVES *

By THOMAS L. PERRY and RICHARD T. JONES †

(From the Division of Chemistry and Chemical Engineering (Contribution no. 2660), California Institute of Technology, Pasadena, Calif. and Pacific State Hospital, Pomona, Calif.)

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This investigation of the concentrations of amino acids in human cerebrospinal fluid (CSF) was stimulated by curiosity as to the mechanism of mental defect in various genetically determined diseases. Studies of serum amino acids and of the pattern of their urinary excretion have in recent years made significant contributions to an understanding of the biochemical etiology of some types of mental deficiency. But it seems likely that in some conditions abnormalities in the biochemistry of the central nervous system may be overlooked if studies are confined to those metabolites excreted in the urine or to those circulating in the blood. Because the CSF is not merely an ultrafiltrate formed by way of the choroid plexuses, but is the product of an exchange between the blood and the central nervous system, examination of this fluid may sometimes yield important clues to deranged cerebral metabolism. In the recently discovered disorder argininosuccinicaciduria (1), for instance, the concentration of argininosuccinic acid has been found to be greater in CSF than in plasma, thus suggesting that the brain rather than some other organ is the site of the metabolic error.

Recent reports concerning the amino acids found in normal human CSF are confusing. Different investigators have found as few as seven to as many as 21 amino acids present. Using paper chromatographic techniques, quantitative estimation of the amino acids in CSF has been attempted by Torre, Scarzella and Zanalda (2), Porcellati and Kemali (3), Walker, Telles and Pastore (4), and by Logothetis (5). Huisman (6) made use of quantitative column chromatography for the determination of amino acids in CSF. Glutamine concentrations in CSF have been estimated by hydrolysis of glutamine to ammonia, and subsequent colorimetric determination of ammonia (7–9). The most recent studies by Knauff, Zickgraf and Mialkowsky (10, 11) have used combined high voltage paper electrophoresis and paper chromatography for the qualitative identification of amino acids in CSF. They found 25 ninhydrin-positive substances, of which they were able to identify 21. There has been a lack of agreement among investigators, in particular concerning the normal occurrence of γ-aminobutyric acid, cystine, proline, and methionine in CSF.

Most studies to date have failed to show differences between the amino acid pattern of normal CSF and of that found in neurological disease. Exceptions are the reports of high CSF levels of argininosuccinic acid in argininosuccinic-aciduria (1), of phenylalanine in phenylketonuria (12), of glutamine in hepatic coma (7–9), and of aspartic acid in hereditary cerebellar ataxia (13).

The development of the automatic amino acid analyzer (14) has made possible more accurate and rapid measurement of a number of ninhydrin-positive substances in physiological fluids. In the present study this instrument was used to determine the concentrations of amino acids in normal pooled CSF. A preliminary investigation was also made of possible differences in amino acids in the CSF of patients suffering from a variety of genetically determined diseases involving mental defect.

MATERIALS AND METHODS

Pooled CSF from adults was used in the study of amino acid concentrations in normal individuals. Ideally it would have been desirable to study individual CSF specimens, and to obtain the fluid from children, since most of the mental defectives studied were children; but there seemed no justification for collecting CSF from normal children, nor for collecting sufficient fluid from individual normal adults to make possible individual amino acid determinations. Accordingly, three pools of

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TABLE I

Mental defectives whose CSF was studied

Patient no.	Age	Sex	No. of lumbar punctures to secure specimen	Clinical diagnosis	Remarks
	yrs				
1	10	M	2	Phenylketonuria } Siblings	
2	11½	F	2	Phenylketonuria }	
3	26	F	2	Phenylketonuria	
4	2	M	2	Tay-Sachs disease	
5	2½	M	2	Tay-Sachs disease	
6	2½	M	2	Tay-Sachs disease	
7	54	M	1	Huntington's chorea	Symptoms for 1¼ years
8	53	F	1	Huntington's chorea	Symptoms for 14 years
9	54	F	1	Huntington's chorea	Symptoms for 17 years
10	12⅓	M	2	Hurler's syndrome* } Siblings	
11	14½	M	2	Hurler's syndrome* }	
12	10½	M	2	Hurler's syndrome*	
13	13½	M	1	Tuberous sclerosis	
14	19⅓	F	2	Tuberous sclerosis	
15	8½	F	2	Mongolism	
16	12	M	2	Mongolism	
17	16	M	2	Schilder's disease	
18	4⅓	M	1	Cerebro-oculo-renal disease	

* No corneal infiltration.

CSF were collected from young women in labor at the time of administering spinal anesthesia just prior to delivery. The subjects were not necessarily in the postabsorptive state. Three to five ml of CSF was obtained by lumbar puncture from each subject, and each of the three pooled specimens contained fluid from 10 to 15 subjects. CSF specimens which were bloody were discarded and not added to the pooled CSF. The pooled CSF was kept frozen at −20° C until used.

CSF specimens were obtained from 18 mentally defective patients by lumbar puncture. These patients also were not necessarily in the postabsorptive state. Fluid specimens which were bloody were discarded, and CSF was frozen immediately and kept at −20° C until used. In 5 patients a single CSF specimen was studied, while in the remaining 13 patients two specimens were obtained on separate occasions and were then pooled. This was necessary because approximately 20 ml of fluid is required for accurate determination of all amino acids present, and it seemed unwise to withdraw this volume of fluid on one occasion from a child. The genetically determined conditions studied were: phenylketonuria, Tay-Sachs disease, Hurler's syndrome (gargoylism), Huntington's chorea, mongolism, tuberous sclerosis, Schilder's disease, and the cerebro-oculo-renal disease of Lowe (15). Table I summarizes the pertinent clinical data for the patients studied.

CSF specimens were deproteinized by ultrafiltration through a cellophane membrane at 5° C over a period of 24 to 36 hours. The pooled CSF specimens were deproteinized after several months' storage at −20° C. Some of the individual CSF specimens from mentally defective patients were deproteinized immediately, while others were first stored at −20° C for as long as 3 months. After being deproteinized, specimens were desalted electrolytically in an electric desalter,[1] using a maximum current of 0.6 a. Each specimen was then divided into 3 fractions, and these were individually taken to dryness under vacuum on a rotating evaporator with the use of gentle heat (not over 40° C). The 2 larger fractions, usually representing 9 or 10 ml of original CSF each, were applied to the 150-cm and 50-cm columns, respectively, of the automatic amino acid analyzer. The third fraction, usually representing 2.5 ml of original CSF, was hydrolyzed with 2 N HCl at 110° C for 2 hours in an evacuated sealed tube, and was applied to the 150-cm column of the analyzer.

The analyses were performed with a Spinco automatic amino acid analyzer, model 120, under the conditions described by Spackman, Stein and Moore (14). The 30° to 50° C systems were employed for the determination of amino acids and derivatives in the unhydrolyzed portions of each CSF specimen. The hydrolyzed portions were analyzed only on the neutral and acidic (150-cm) column, and most of the latter analyses were carried out using the 50° C system.

Identification of the ninhydrin-positive components found in CSF was based upon comparison with ion exchange chromatograms prepared from solutions of authentic amino acids and related compounds. In addition, two-dimensional paper chromatography carried out in this laboratory prior to this investigation had demonstrated the presence in pooled human CSF of the following amino acids: alanine, arginine, aspartic acid, glutamic acid, glutamine, glycine, histidine, isoleucine-leucine,

[1] Research Specialties Co., 200 S. Garrard Blvd., Richmond, Calif.

lysine, phenylalanine, serine, taurine, threonine, tryptophan, tyrosine, and valine.

Calculations of the amounts of amino acids present in each analyzed specimen of CSF were made in the standard manner as described for the automatic recording equipment (14). With this technique it is possible to measure amino acids with a precision in the order of 100 ± 3 per cent for loads of 0.1 to 3.0 μmole of each amino acid. Color values for the common amino acids were based on analyses of standard amino acid solutions supplied by the Spinco Company. Color values for amino acids and related compounds not present in the Spinco standards (i.e., taurine, urea, citrulline, α-amino-*n*-butyric acid, cystathionine, ornithine and ethanolamine) were estimated by using the color values given by Spackman and associates (14), corrected by multiplying them by the ratio of the leucine color value determined for the present instrument to that reported for leucine by the above investigators. All unidentified compounds present in CSF were calculated as if they were leucine, using the ninhydrin color value of leucine.

In addition to the analysis of each CSF specimen on the automatic amino acid analyzer, 2 of the 3 pooled specimens of normal CSF were chromatographed one-dimensionally on Whatman no. 1 paper in isopropanol: concentrated ammonia: water (8:1:1). The sheets were developed with *p*-dimethylaminocinnamaldehyde, and the tryptophan spots which were detected were compared visually with graded mounts of authentic tryptophan which had been chromatographed simultaneously. In this manner the tryptophan content of these specimens was estimated, since this amino acid was difficult to measure in eluates from the amino acid analyzer. The quantities of CSF obtained from the defective patients were insufficient to permit estimation of tryptophan by this technique.

RESULTS

The concentrations of the amino acids found in the three pools of CSF from normal adults as well as in the individual fluids from 18 mental defectives

TABLE II

Amino acid content of normal pooled CSF

Amino acid	Pool A	Pool B	Pool C	Mean	Mean
	μmoles per 10 ml		*μmoles per 10 ml*		*mg per 100 ml*
Unknown no. 1*	0.026	0.026	0.023	0.025	
Unknown no. 2*	0.028	0.016	0.055	0.033	
Unknown no. 3*	0.035	0.041	0.037	0.038	
Unknown no. 4*	0.056	0.064	0.039	0.053	
Unknown no. 5*	0	†	‡	‡	
Taurine	0.067	0.051	0.057	0.058	0.07
Urea	26.82	22.46	29.76	26.35	15.81
Unknown no. 6*	†	0.049	0.037	0.043	
Aspartic acid	0.076	0.016	‡	0.031	0.04
Unknown no. 7*	1.060	0.872	0.280	0.737	
Threonine	0.487	0.437	0.513	0.479	0.57
Serine	0.700	0.428	0.388	0.505	0.53
Asparagine	<0.216	<0.223	<0.234	<0.224	<0.30
Glutamine	1.607	2.244	2.822	2.224	3.25
Proline	0.042	‡	0	0.014	0.01
Glutamic acid	0.088	0.090	0.048	0.075	0.11
Citrulline	‡	‡	0	‡	‡
Glycine	0.194	0.061	0.195	0.150	0.11
Alanine	0.366	0.284	0.321	0.324	0.29
α-Amino-*n*-butyric acid	0.013	0.015	0	0.009	0.01
Valine	0.138	0.169	0.118	0.142	0.17
Cystathionine	‡	‡	‡	‡	‡
Methionine	0.032	0.020	0.028	0.027	0.04
Isoleucine	0.051	0.043	0.039	0.044	0.06
Leucine	0.106	0.089	0.098	0.098	0.13
Tyrosine	0.068	0.076	0.054	0.066	0.12
Phenylalanine	0.094	0.087	0.084	0.088	0.15
Unknown no. 8*	§	0.007	0	0.004	
Ornithine	§	0.042	0.034	0.038	0.05
Ethanolamine	§	0.191	0.066	0.129	0.08
Lysine	§	0.206	0.134	0.170	0.25
Histidine	§	0.148	0.109	0.129	0.20
Tryptophan	§	0.012	0.015	0.014	0.03
Unknown no. 9*	§	0.013	‡	0.007	
Arginine	§	0.105	0.092	0.099	0.17

* Calculated as leucine-equivalents.
† Present on chromatogram in measurable amount, but technical failure prevented quantitation.
‡ Present on chromatogram, but amount insufficient for quantitation.
§ Measurement not attempted.

TABLE III

Amino acid content of CSF of mental defectives (in μmoles/10 ml)

Amino acid	Normal mean (from Table II)	Phenylketonuria			Tay-Sachs disease			Huntington's chorea		
		1	2	3	4	5	6	7	8	9
Unknown no. 1*	0.025	0.016	0.029	0.021	0.016	0.018	0.029	0.020	0.017	0.019
Unknown no. 2*	0.033	†	0.047	0.011	†	0.010	0.021	0.013	0.016	0.013
Unknown no. 3*	0.038	0.019	†	†	†	0.010	†	0.025	0	†
Unknown no. 4*	0.053	0.056	0.042	0.038	0.038	0.050	0.028	0.062	0.044	0.036
Unknown no. 5*	†	0	†	0	0	0	0	0	0	0
Taurine	0.058	0.061	0.065	0.059	0.040	0.036	0.045	0.064	0.058	0.070
Urea	26.35	29.23	20.03	38.58	19.95	24.85	24.10	40.15	46.65	37.20
Unknown no. 6*	0.043	0.049	0	0.054	0.031	0.034	0.025	0.047	0.037	0.031
Aspartic acid	0.031	†	†	0.021	0.013	0.007	†	0.010	†	0.013
Unknown no. 7*	0.737	0.047	0.364	‡	‡	‡	‡	0.503	1.095	‡
Threonine	0.479	0.152	0.136	0.219	0.210	0.177	0.199	0.327	0.298	0.227
Serine	0.505	0.528	0.417	‡	0.308	0.308	‡	0.438	0.430	0.300
Asparagine	<0.224	<0.259	<0.262	<0.184	<0.107	<0.163	<0.136	<0.593	<0.103	<0.169
Glutamine	2.224	<4.733	<1.153	<5.856	2.348	2.396	2.537	4.008	2.265	2.369
Proline	0.014	0	0	0	†	0.042	0.141	0	0	0.033
Glutamic acid	0.075	0.085	2.373	0.018	0.044	0.049	0.015	0.116	0.030	0.016
Citrulline	†	0	0	0.030	†	0.017	0.013	†	0	0.022
Glycine	0.150	0.092	0.078	0.124	0.060	0.060	0.093	0.080	0.059	0.095
Alanine	0.324	0.302	0.180	0.294	0.272	0.283	0.413	0.443	0.298	0.278
α-Amino-n-butyric acid	0.009	0	†	†	0.034	0.048	0.044	0.047	†	0.016
Valine	0.142	0.112	0.119	0.128	0.173	0.172	0.222	0.237	0.109	0.107
Cystathionine	†	0	†	†	†	†	0	†	†	†
Methionine	0.027	0.035	0.023	0.035	0.021	0.028	0.024	‡	0.029	0.028
Isoleucine	0.044	0.035	0.040	0.045	0.056	0.058	0.072	0.081	0.035	0.040
Leucine	0.098	0.088	0.086	0.088	0.116	0.118	0.130	0.187	0.081	0.082
Tyrosine	0.066	0.083	0.160	‡	0.111	0.123	0.112	0.117	0.048	0.068
Phenylalanine	0.088	3.231	2.863	3.851	0.063	0.073	0.077	0.111	0.098	0.077
Unknown no. 8*	0.004	0	0	0	0.006	0.021	0	0.007	0	0
Ornithine	0.038	0.080	0.035	0.112	0.071	0.045	0.040	0.082	0.060	0.080
Ethanolamine	0.129	0.140	0.083	0.108	0.101	0.067	0.044	0.173	0.106	0.144
Lysine	0.170	0.211	0.125	0.212	0.195	0.156	0.174	0.314	0.267	0.218
Histidine	0.129	0.250	0.175	‡	0.094	0.094	0.103	0.126	0.119	0.126
Tryptophan	0.014	§	§	§	§	§	§	§	§	§
Unknown no. 9*	0.007	0.077	0.056	0.050	†	0.010	0	0.019	†	0.017
Arginine	0.099	0.180	0	0.122	0.111	0.129	0.122	0.226	0.225	0.260

* Calculated as leucine-equivalents.
† Present on chromatogram, but amount insufficient for quantitation.
‡ Present on chromatogram in measurable amount, but technical failure prevented quantitation.
§ Measurement not attempted.

are presented in Tables II, III and IV. Figures 1, 2 and 3 present in graphic form the chromatographic analysis of one of the pooled specimens of normal CSF.

The following ninhydrin-positive substances were regularly found in the CSF of normal individuals and of the mental defectives studied: taurine, urea, aspartic acid, threonine, serine, asparagine, glutamine, glutamic acid, glycine, alanine, α-amino-n-butyric acid, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ornithine, ethanolamine, ammonia, lysine, histidine, and arginine. Found less frequently and usually in small amounts were citrulline and proline. Cystine, γ-amino-butyric acid and argininosuccinic acid were not detected in any CSF specimen. Tryptophan was not estimated in the chromatograms of the automatic amino acid analyzer, but was found in small amounts when searched for by the paper chromatographic technique described. In most CSF specimens a very small rise in absorbance was noted at the point where cystathionine is eluted from the 150-cm column, just after the artifact produced on the chromatogram by the buffer change from pH 3.25 to pH 4.25. Whether or not this represents a trace of cystathionine in the CSF specimens studied is not clear.

In addition, six unidentified peaks were regularly encountered on the chromatograms of unhydrolyzed CSF, and two further unidentified peaks were noted in some specimens. Of the unidentified ninhydrin-positive substances regularly present in CSF, Unknown no. 1 emerged from the column at the position of phosphoserine and phosphothreonine, and Unknown no. 4 emerged at the position of phosphoethanolamine. In most CSF specimens prior to hydrolysis, and in all specimens after hydrolysis, an unexpected peak was encountered on chromatograms after urea and before aspartic acid. This substance, Unknown no. 6, was usually eluted from the 150-cm column at 101 ml effluent volume in the 30° to 50° C system, and was eluted at 91 ml when the column was operated at 50° C. In the same two systems, aspartic acid was usually eluted at 135 and at 110 ml, respectively. Unknown no. 6 had the same rate of travel on the column as the "erythro" form of β-hydroxyaspartic acid, and *allo*-γ-hydroxyglu-

tamic acid. Unknown no. 8, present in some CSF specimens, was eluted from the 50-cm column at the position of hydroxylysine, while Unknown no. 9, present in all fluids studied, was eluted from this column at the position of carnosine.

Since asparagine and glutamine emerge from the column at the same effluent volume and give a combined peak on the chromatogram, values for these two amino acids were estimated from the increase in aspartic and glutamic acids, respectively, after acid hydrolysis of CSF. Serine was usually poorly separated, if at all, from asparagine and glutamine, and serine levels were therefore usually measured after hydrolysis.

After acid hydrolysis of CSF, the following changes were noted. Glutamic acid showed the greatest increase over the concentration found in the unhydrolyzed CSF, but there was also a marked increase in aspartic acid. Unknown no. 6 showed a three- to tenfold increase. The amount of glycine present was usually doubled. Methionine disappeared and was replaced by methionine sulfoxides. Urea was regularly decreased in amount. The values for alanine, α-amino-*n*-butyric acid, valine, isoleucine, leucine, tyrosine and phenylalanine were not altered by hydrolysis, and cystine was found in no specimen following hydrolysis.

Finally, a large and highly variable amount of an additional unidentified ninhydrin-positive substance, Unknown no. 7, was found on chromatograms after hydrolysis. It was eluted from the column just before threonine at 50° C and just after serine at 30° to 50°C. This substance could not be detected in CSF prior to hydrolysis, possibly because of incomplete separation from serine, asparagine and glutamine. It was itself resistant to hydrolysis in 6 N HCl at 110° C for 30 hours. It is presumed that this unidentified substance is an amino acid or an amino acid derivative, perhaps normally present in CSF, either partly or entirely, in a conjugated form.

It is likely that in most of the CSF specimens studied the marked increase in glutamic acid after hydrolysis could be accounted for entirely by the decomposition of glutamine. A possible exception

TABLE IV

Amino acid content of CSF of mental defectives (in μmoles/10 ml)

Amino acid	Normal mean (from Table II)	Hurler's syndrome			Tuberous sclerosis		Mongolism		Schilder's disease	Cerebro-oculo-renal disease
		10	11	12	13	14	15	16	17	18
Unknown no. 1*	0.025	0.022	0.013	0.018	0.024	0.022	0.024	0.022	0.017	0.007
Unknown no. 2*	0.033	0.017	0.008	†	0.032	†	†	0.039	0.028	†
Unknown no. 3*	0.038	†	†	0.012	†	0.022	0.032	0.028	0.018	0.009
Unknown no. 4*	0.053	0.023	0.036	0.030	0.036	0.060	0.085	0.037	0.037	0.040
Unknown no. 5*	†	0	0	0	0	0	0	0.066	0	0
Taurine	0.058	0.071	0.030	0.050	0.053	0.068	0.077	0.067	0.059	0.028
Urea	26.35	22.00	16.00	29.30	26.34	31.30	29.20	29.72	39.14	42.60
Unknown no. 6*	0.043	0.035	0.039	0.049	0	0.040	0.045	0	0	0.021
Aspartic acid	0.031	†	†	0.004	†	0.009	0.008	0.023	†	0.015
Unknown no. 7*	0.737	0.726	1.399	0.350	‡	0	0.116	‡	‡	0.357
Threonine	0.479	0.309	0.429	0.242	0.182	0.257	0.174	0.262	0.223	0.063
Serine	0.505	0.363	0.398	0.259	0.418	0.343	0.378	‡	‡	0.201
Asparagine	<0.224	<0.136	<0.129	<0.017	<0.235	<0.127	<0.265	<0.096	<0.150	<0.165
Glutamine	2.224	2.996	3.002	3.100	1.508	2.315	3.068	1.454	2.932	2.440
Proline	0.014	0	0	0	0	0	0	†	0	0
Glutamic acid	0.075	0.059	0.024	0.118	0.574	0.067	0.124	0.274	0.050	0.030
Citrulline	†	0.023	0.020	†	0	0	0	0	†	†
Glycine	0.150	0.066	0.074	0.053	0.043	0.076	0.056	0.111	0.070	0.110
Alanine	0.324	0.175	0.232	0.214	0.184	0.170	0.250	0.252	0.280	0.228
α-Amino-*n*-butyric acid	0.009	0.014	†	0.009	†	0.015	0.010	0.022	0.030	†
Valine	0.142	0.119	0.099	0.096	0.103	0.098	0.144	0.156	0.185	0.118
Cystathionine	†	†	†	0	0	†	†	†	†	0
Methionine	0.027	0.044	0.030	0.020	0.018	0.020	0.022	0.028	0.030	0.014
Isoleucine	0.044	0.036	0.031	0.031	0.035	0.035	0.049	0.054	0.052	0.049
Leucine	0.098	0.083	0.065	0.072	0.088	0.082	0.120	0.130	0.118	0.088
Tyrosine	0.066	0.056	0.054	0.059	0.085	0.078	0.084	0.082	0.094	0.046
Phenylalanine	0.088	0.077	0.075	0.067	0.085	0.091	0.097	0.125	0.099	0.065
Unknown no. 8	0.004	0	†	0.011	0	0.007	†	0	0	0
Ornithine	0.038	0.025	0.059	0.085	0.063	0.077	0.063	0.048	0.057	0.108
Ethanolamine	0.129	0.082	0.086	0.107	0.061	0.205	0.179	0.046	0.061	0.132
Lysine	0.170	0.069	0.163	0.145	0.104	0.165	0.228	0.181	0.130	0.185
Histidine	0.129	0.137	0.165	0.151	0.097	0.093	0.111	0.098	0.110	0.095
Tryptophan	0.014	§	§	§	§	§	§	§	§	§
Unknown no. 9	0.007	†	0.034	0.028	0.035	0.017	0.036	0.029	0.020	†
Arginine	0.099	0.133	0.196	0.148	0	0.027	0.149	0	0.165	0.135

* Cacluated as leucine-equivalents.
† Present on chromatogram, but amount insufficient for quantitation.
‡ Present on chromatogram in measurable amount, but technical failure prevented quantitation.
§ Measurement not attempted.

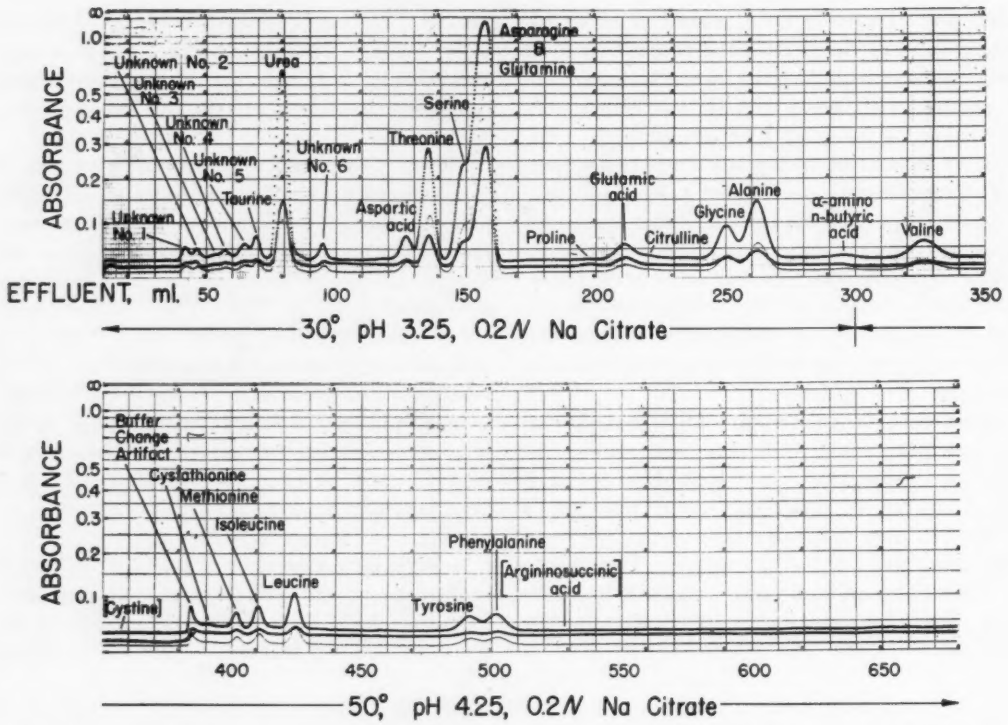

FIG. 1. CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS IN 10 ML OF NORMAL CSF APPLIED TO A 150-CM COLUMN. Compounds enclosed in brackets were not detected in any specimens studied.

is the case of the CSF specimens from phenylketonurics, where the fluid may have contained phenylacetylglutamine, which on hydrolysis would release glutamic acid. The increase in aspartic acid, on the other hand, might result either from the hydrolysis of its corresponding amide, or from the hydrolysis of N-acetylaspartic acid. This latter amino acid conjugate has been found in brain tissue in relatively high concentration (16), and might well be present in CSF. Since it is a ninhydrin-negative compound, it would not be detected in unhydrolyzed CSF. The values given for asparagine in Tables II, III and IV, therefore, are maximum values and may represent either asparagine or a combination of both compounds. The increase in glycine regularly noted following hydrolysis was probably due to the splitting of hippuric acid present in the CSF. The absence of cystine and the failure of the α-amino-*n*-butyric acid peak to increase after acid hydrolysis provides evidence that glutathione and ophthalmic acid were absent from the CSF specimens studied.

In general the concentrations of amino acids present in the CSF of the eight forms of mental defect studied did not vary appreciably from those found in pooled CSF from normal adults. The only exception to this was the expected high concentration of phenylalanine found in the CSF of the three phenylketonuric patients. This ranged from 4.74 to 6.37 mg per 100 ml of CSF, and was similar to that found by Borek, Brecher, Jervis and Waelsch (12) using a microbiological method to determine phenylalanine in the CSF of phenylketonurics. The level of α-amino-*n*-butyric acid was relatively high in the CSF of the three patients having Tay-Sachs disease, and two of these also showed appreciable amounts of proline, but the significance of these variations is doubtful.

DISCUSSION

The amino acid contents of CSF as determined by other investigators is summarized in Table V and this is compared with that found in the present investigation. Proline, citrulline, asparagine

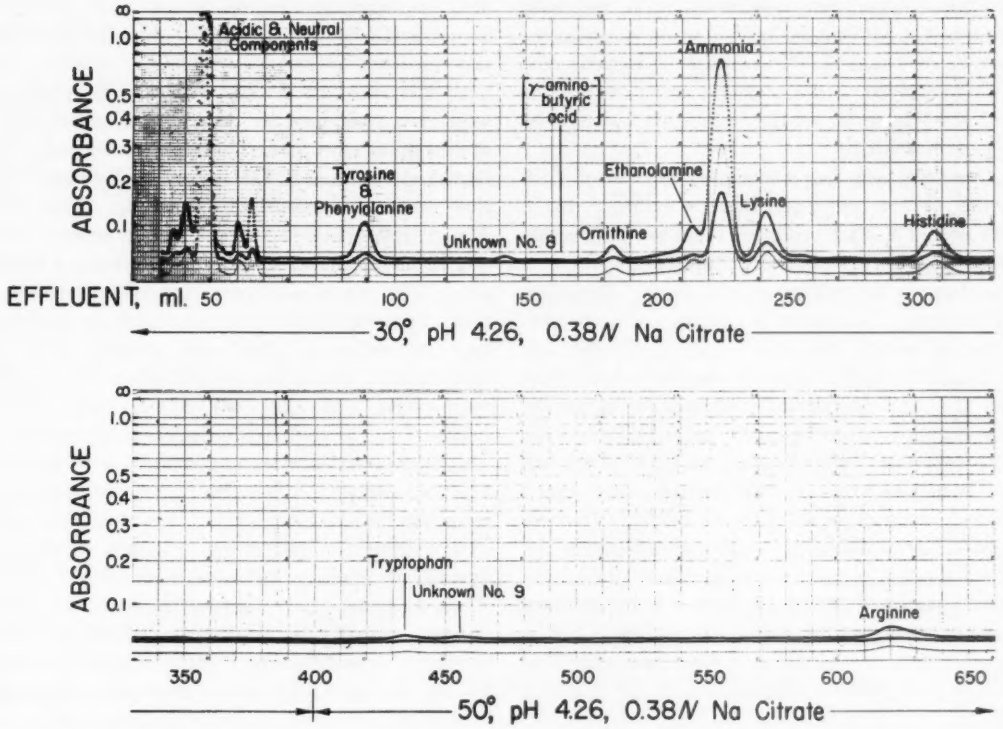

FIG. 2. CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS IN 10 ML OF NORMAL CSF APPLIED TO A 50-CM COLUMN. γ-Aminobutyric acid was not detected in any specimen studied.

and cystathionine have not been previously reported as components of CSF. Walker and coworkers (4) found proline and methionine only in fluids which contained red blood cells. In the present study methionine was found in a measurable amount in all except one CSF specimen, and in this fluid it was replaced by methionine sulfoxides. Proline and citrulline were found in a number of the fluids examined. It is apparent that these three amino acids are normal constituents

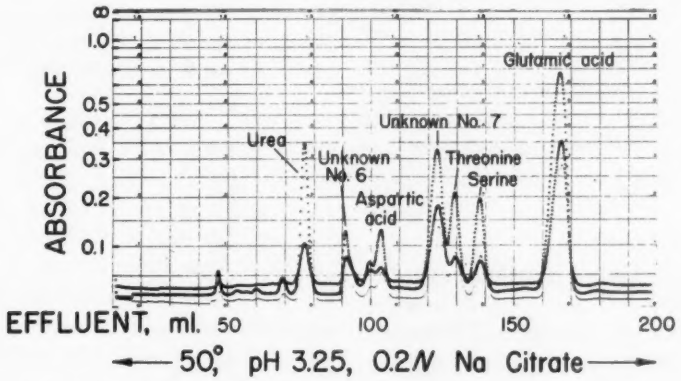

FIG. 3. CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS IN 5 ML OF NORMAL CSF WHICH HAD BEEN HYDROLYZED WITH 2 N HCl FOR 2 HOURS AT 110° C BEFORE APPLICATION TO A 150-CM COLUMN.

of CSF. Asparagine is believed to be regularly present in CSF, but its identification is presumptive, being based on the increase in aspartic acid after hydrolysis. In any event, either asparagine or N-acetylaspartic acid or both must normally be present in CSF.

Ornithine was found in each specimen of CSF in the present study, and there is evidence that it was not an artifact produced from the destruction of arginine during electrolytic desalting. Arginine was lost to a variable extent during desalting. In one pooled specimen of normal CSF, chromatography before and after desalting showed that although all of the arginine had been lost, there had been no concomitant increase in ornithine. The arginine values listed in Table II for normal CSF represent determinations on CSF which had not been desalted. Values for all other amino acids, and for arginine in the CSF of the 18 mentally defective patients, were determined on desalted fluids. Subsequent experiments have indicated that good chromatograms can be obtained without desalting, if the deproteinized CSF is first adjusted to pH 2.2 with HCl before application to the column. Elimination of the desalting step in future studies of amino acids in CSF would avoid the partial or complete loss of arginine incurred in the present study.

Cystine was not found in any CSF specimen studied. It may be that cysteine and cystine were originally present in CSF, only to have been lost during storage at − 20° C, or during the relatively slow deproteinization method used. Stein and Moore (17) have reported that the cystine content of plasma decreases during prolonged storage at − 20° C, possibly as a result of oxidative formation of intermolecular bridges involving cysteine and the sulfhydryl groups of proteins. In future studies, immediate deproteinization of CSF with picric acid after its collection at lumbar puncture might lead to the detection of cystine.

γ-Aminobutyric acid was not detected in any CSF specimen. Although this amino acid has been reported as a normal constituent of CSF (5, 11), Ludewig (18), using a paper chromatographic technique, was unable to detect it in fluids obtained from 26 patients suffering from various neurological diseases. Despite its relatively high concentration in brain tissue, γ-aminobutyric acid apparently is not present in detectable

TABLE V

*Comparative study of amino acids in normal human CSF**

Authors Reference no.	Torre *et al.* (2)	Porcellati and Kemali (3)	Walker *et al.* (4)	Logothetis (5)	Huisman (6)	Knauff *et al.* (10, 11)	Various authors (7–9)	Present study
Method used	Paper chromatography	Paper chromatography	Paper chromatography	Paper chromatography	Column chromatography	Paper electrophoresis and chromatography	Chemical determination	
No. of subjects	10		26	52	1 pooled CSF	20	131	
Alanine	0.08	0.84	0.11	0.15	0.15	†		0.29
α-Amino-n-butyric acid				0.02		†		0.01
γ-Aminobutyric acid				0.04		†		0
Arginine	0.08			0.16	0.10	†		0.17
Asparagine								0.30
Aspartic acid			0.04	0.11	0.10	†		0.04
Citrulline								‡
Cystine	0.21				0.05			§
Cystathionine								‡
Ethanolamine						0.10		0.08
Glutamic acid	1.04	1.22	0.06	0.09	3.35	†		0.11
Glutamine			0.71	0.30		†	5 to 22	3.25
Glycine	0.11	0.48	0.10	0.09	0.10	†		0.11
Histidine	0.22				0.05	†		0.20
Isoleucine					0.05	†		0.06
Leucine	0.11	0.24	0.14	0.14	0.05	†		0.13
Lysine				0.17	0.20	†		0.25
Methionine					0.05	†		0.04
Ornithine						†		0.05
Phenylalanine	0.09	0.18	0.21	0.18	0.15	†		0.15
Proline								0.01
Serine			0.16	0.22	0.10	†		0.53
Taurine						†		0.07
Threonine			0.13	0.12	0.30	†		0.57
Tryptophan	0.26					†		0.03
Tyrosine	0.11	0.22	0.08	0.16	0.10	†		0.12
Valine	0.14	0.36	0.07	0.12	0.15	†		0.17

* Values are expressed in mg per 100 ml CSF, and except where indicated are mean values.
† Substance present, but concentration not measured.
‡ Present in trace amounts.
§ Cystine not determined.

amounts in CSF. It is also clear from the present study that argininosuccinic acid and glutathione are normally absent from CSF.

The finding of nine unidentified peaks on chromatograms of CSF had not been expected. One of these, Unknown no. 7, was usually present in relatively large amounts, and in a number of fluids exhibited an apparent concentration greater than that of serine and threonine. An unidentified ninhydrin-positive substance, emerging from the column of the amino acid analyzer at the same point as Unknown no. 6 in the present study, has been noted by Moore, Tallan and Stein in human blood plasma, liver and brain extracts (19, 20). Further studies are planned to attempt identification of these unknown compounds.

The relative concentrations of the various amino acids in CSF and in postabsorptive plasma in normal adults are compared in Table VI. The data for plasma (17, 21) were obtained by a column chromatographic technique similar to that employed in this study for the quantitation of amino acids in CSF. It will be noted that glutamine is the predominant amino acid both in plasma and in CSF. Most of the amino acids present in CSF occur in concentrations only 5 to 15 per cent of those found in plasma. In the case of proline, the disparity between CSF and plasma levels is much more marked. On the other hand, glutamine, asparagine, serine and threonine occur in CSF in close to one-half their concentration in plasma. Aspartic acid levels are about equal between the two fluids. Ethanolamine is present in greater concentration in the CSF than in plasma. This would suggest that the CSF is not a simple mirror of conditions prevailing in the blood stream, but also reflects in part the chemical milieu of the brain.

Although the measurement of amino acid concentrations in the CSF of several genetically determined diseases in this study failed to provide clues to the mechanism of the mental defect characteristic of each disease, this approach may be worth applying to other neurological disorders. When patients become available, it would be of interest to measure amino acid concentrations in the CSF in conditions such as maple syrup urine disease and cystathioninuria. In the former condition, it is anticipated that CSF concentrations of leucine, isoleucine, valine and methionine will be found to be elevated for the same reason that phenylalanine has been found elevated in the CSF of phenylketonurics. In the latter condition, the finding of a larger amount of cystathionine in CSF than in the plasma would help to localize the site of the metabolic error. At the same time, it must be realized that the failure to detect unusual amino acid concentrations in CSF does not eliminate the possibility that a given form of mental defect may be caused by deranged cerebral amino acid metabolism; for the amino acid in question may, like γ-aminobutyric acid, fail to accumulate in the CSF in spite of a high concentration in brain tissue.

TABLE VI

*Relative concentration of amino acids in normal adult human plasma and CSF**

Amino acid (or related compound)	Plasma (17, 21)	CSF
Alanine	3.41	0.29
α-Amino-*n*-butyric acid	0.30	0.01
Arginine	1.51	0.17
Asparagine	0.58	0.30
Aspartic acid	0.03	0.04
Citrulline	0.50	†
Cystathionine		†
Cysteine	0.40	‡
Cystine	1.00	‡
Ethanolamine		0.08
Glutamic acid	0.70	0.11
Glutamine	8.30	3.25
Glycine	1.54	0.11
Histidine	1.15	0.20
Isoleucine	0.89	0.06
Leucine	1.69	0.13
Lysine	2.72	0.25
Methionine	0.38	0.04
1-Methylhistidine	0.11	
3-Methylhistidine	0.08	
Ornithine	0.72	0.05
Phenylalanine	0.84	0.15
Proline	2.36	0.01
Serine	1.12	0.53
Taurine	0.55	0.07
Threonine	1.39	0.57
Tryptophan	1.11	0.03
Tyrosine	1.03	0.12
Valine	2.88	0.17

* Mean values expressed in mg per 100 ml plasma or CSF.
† Present in trace amounts.
‡ Cysteine and cystine not determined.

SUMMARY

Concentrations of amino acids have been determined in pooled normal human cerebrospinal fluid (CSF) using the automatic amino acid analyzer. These have been compared with values obtained by other investigators using different techniques, as well as with the concentrations of amino acids found in human postabsorptive plasma.

Twenty-two amino acids were regularly present in CSF, and three others were sometimes present. Cystine and γ-aminobutyric acid, which have been reported as constituents of CSF, were not detected. Seven unidentified ninhydrin-positive substances were regularly found in CSF, one of them in relatively large amounts.

Amino acid concentrations were similarly measured in the CSF of patients having genetically determined diseases characterized by mental defect. These disorders included phenylketonuria, Tay-Sachs disease, Hurler's syndrome, Huntington's chorea, tuberous sclerosis, mongolism, Schilder's disease, and the cerebro-oculo-renal disease of Lowe. Except for the finding of greatly elevated concentrations of phenylalanine in the CSF of the phenylketonurics studied, there were no significant deviations in these patients from the amino acid concentrations found in pooled normal CSF. The possible usefulness of applying this technique to other neurological disorders is discussed.

ACKNOWLEDGMENTS

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THYROXINE DISPLACEMENT FROM SERUM PROTEINS AND DEPRESSION OF SERUM PROTEIN-BOUND IODINE BY CERTAIN DRUGS

By J. WOLFF, M. E. STANDAERT AND J. E. RALL

(From the Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.)

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A number of compounds such as 2,4-dinitrophenol,[1] salicylate, tetrachlorothyronine, and diphenylhydantoin have been shown to lower the protein-bound iodine level of the blood (1–5). In the case of DNP and salicylate, it was concluded that they acted in part via an inhibition of the pituitary TSH mechanism and partly by an accelerated loss of T_4 from the circulation (2, 6). A likely site of action was, therefore, expected to involve binding of T_4 to serum proteins.

The ability of various analogs of thyroxine to bind to proteins of serum has been extensively studied with electrophoretic techniques. At least three serum proteins and perhaps four have been described which bind thyroxine (7). Less direct methods of studying this binding of the thyroid hormones consist of measurement of the "uptake" of triiodothyronine by erythrocytes (8) and the rate of dialysis of T_4 through a cellophane membrane (9). By these techniques several compounds have been found that appear to alter the binding of T_4 to serum proteins when studied by these techniques but if whole serum is used, the effect cannot be localized to specific proteins. Thus it has been suggested from dialysis studies that salicylates and DNP act by inhibiting T_4 binding to serum proteins. Electrophoretic studies in barbital buffer of sera containing salicylate failed to show any influence on the distribution of T_4 between TBG and albumin, nor was there any change in the T_4 binding capacity measured under these conditions (2). Since then it has been shown that barbital buffer inhibits T_4 binding to prealbumin (10) and Ingbar has stated that salicylate could interfere with T_4 binding in electrophoretic systems in buffers other than barbital (11). It was therefore desired to examine T_4 binding in the presence of these four agents in a system where prealbumin binding of T_4 could be observed. Furthermore, the possible correlation between the effect of inhibition of thyroxine binding to TBPA and to TBG by various drugs with the *in vivo* effect of these drugs on the PBI was studied.

METHODS

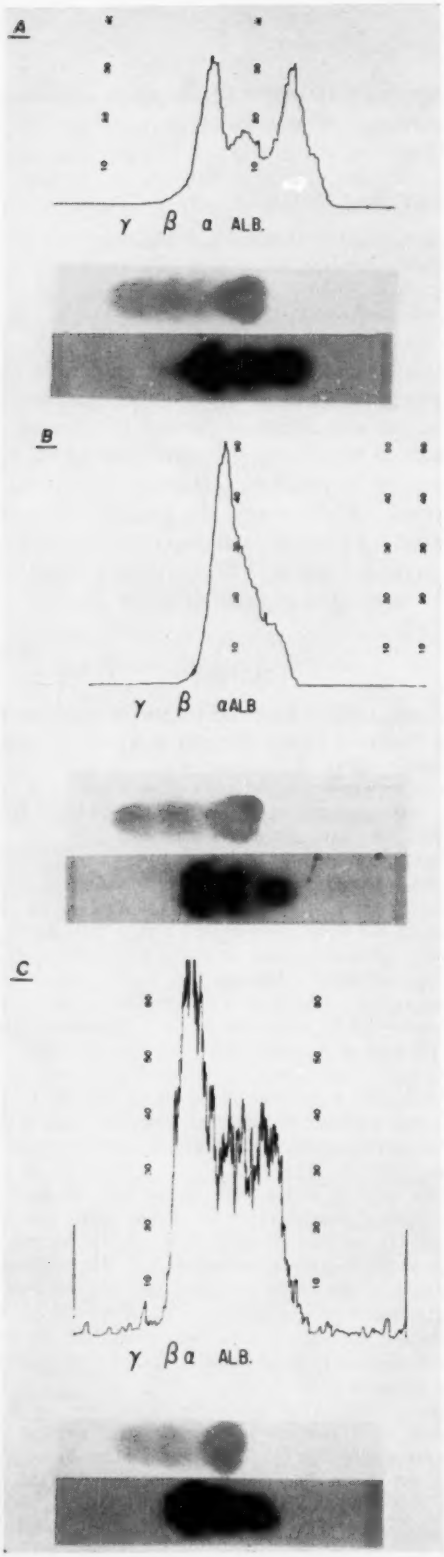
A single batch of pooled normal human serum obtained from the Baxter Laboratories was used in all the studies. Butanol solutions of thyroxine were evaporated to dryness *in vacuo* prior to the addition of serum. The mixtures were incubated at 4° C for at least 24 hours before use and were kept in the cold until used.

Electrophoresis in 0.12 M ammonium carbonate buffer, pH 8.4, was performed at room temperature on 3.75 × 49 cm strips of Whatman no. 3 MM filter paper. The edges of the strips were coated with a thin border of paraffin. The strips were saturated with buffer and suspended horizontally between two plastic clamps in a closed system. The strips were equilibrated for at least 1 hour before the serum was applied. The anodal chamber received an excess of buffer solution just before the current was turned on. Thirty μl of serum was added approximately 8 cm from the anode just before the voltage was applied. In nearly all cases the reverse flow technique was employed (12) with a constant voltage of 80 v.

Two types of studies were carried out. In the first, the inhibitor was added to the serum which was then run in reverse flow electrophoresis. In the second, we followed the suggestion of Ingbar (11) that addition of salicylate to the buffer permitted the demonstration of interference in protein-binding of T_4. Conventional electrophoresis was used in some cases to rule out the presence of unbound T_4 in the albumin (origin) area in reverse flow electrophoresis.

After a run of 17 hours the strips were air-dried, scanned on a continuous recording strip counter, and radioautographed on No-Screen X-ray film. Strips were realigned on the film and the radioactive fractions cut

[1] The following abbreviations have been used: DNP = 2,4-dinitrophenol; PBI = protein-bound iodine; T_4 = thyroxine; TBG = thyroxine-binding globulin of the inter-alpha zone in paper electrophoresis; TBPA = thyroxine-binding prealbumin; TCT = DL-tetrachlorothyronine; TSH = thyroid-stimulating hormone.



out, using the radioautograph as a guide to their exact location. The filter paper segments were counted in a well scintillation counter. A duplicate strip was stained with bromphenol blue to show the protein location. All results are averages of duplicate or quadruplicate determinations and are reported as fractions of the total thyroxine on each protein fraction.

It is felt that with the reverse flow technique direct counting offered greater accuracy than quantitation of the strip counter recordings. This is especially true in instances where the various agents have displaced all but trace amounts of thyroxine from a binding site. Such quantities are difficult to measure accurately on the strip counter.

For calculations, serum PBI measured by a modification of the Zak procedure (performed by the Boston Medical Laboratories) was assumed to consist entirely of thyroxine. Concentrations of T_4 listed in Table I have been corrected for the endogenous T_4 of the serum used. Labeled thyroxine was obtained from Abbott Laboratories and chromatographed before use; when necessary it was purified until better than 90 per cent pure (12). DNP and sodium salicylate were reagent grade and TCT was a generous gift of Dr. Jacob Lerman. When drugs were added directly to the ammonium carbonate buffer, the pH was adjusted after addition of the drug.

Male guinea pigs of the NIH strain were used. In experiment III (Table II) thyroidectomy was checked by thorough examination of the tracheal region, and all animals suspected of incomplete thyroidectomy were discarded. They were then divided into three groups: an uninjected control group, a thyroxine-injected control group, and a group receiving both thyroxine and TCT. The thyroxine treatment was started 8 days after thyroidectomy and was continued for 12 days. During the last 5 days the third group of guinea pigs received, in addition, subcutaneous injections of TCT. All animals were exsanguinated 3 to 4 hours after the last injection.

RESULTS

Initial experiments, in which salicylate or DNP was added to the serum before electrophoresis, showed that both these agents tended to displace T_4 from TBPA onto TBG. However, experiments in which these agents were added to the buffer gave more reproducible results, and most of the studies presented here will deal with the latter technique.

FIG. 1. THE EFFECT OF 2,4-DINITROPHENOL ON THE ELECTROPHORETIC DISTRIBUTION OF RADIOACTIVE THYROXINE (0.28 μG PER ML OR 0.36 μM) IN HUMAN SERUM. In this and succeeding figures a record of the radioactivity is above the stained paper strip and a radioautograph of the paper strip is below it. A) Control serum; B) 1×10^{-4}M dinitrophenol in the buffer; C) 1×10^{-4}M dinitrophenol added to serum.

It can be seen from Figures 1 and 2 that the addition of 1×10^{-4} M DNP or 3×10^{-3} M Na salicylate to human serum containing 3.6×10^{-7} M T_4 caused significant displacement of T_4 from the prealbumin peak to TBG. This effect was more marked when DNP or salicylate had been added to the $(NH_4)_2CO_3$ buffers (Figures 1B and 2B as compared with Figures 1C and 2C). At 2.7×10^{-7} M T_4 (Figure 3C) there was practically no radioactivity left in the prealbumin area. At these low T_4 concentrations most of the T_4 displaced from TBPA was bound by TBG. In the presence of higher amounts of T_4, the TBG became saturated and the displaced T_4 was bound to albumin (see Table I). That there was no displacement from albumin was ascertained in studies with standard paper electrophoresis in which albumin is permitted to migrate from the origin. Thyroxine stayed with the albumin and no radioactivity was found at the origin. Displacement of T_4 from prealbumin as a function of DNP or salicylate concentration is also demonstrated in Table I. Displacement by salicylate could still be demonstrated at 3×10^{-4} M, a level easily attained during salicylate therapy (4.1 mg per 100 ml), and was marked at 1 to 3×10^{-3} M levels which have been shown to be accompanied by lowering of the PBI (2).

TCT also could displace T_4 from serum proteins, in this case from TBG (Figures 3A and 3B). Some displacement of T_4 from TBG can be observed at TCT levels as low as 10^{-7} M. The displacement can also be demonstrated when the compound is added directly to serum. Comparison of Figures 3B and 3C reveals the striking difference between TCT and salicylate in the electrophoretic distribution of T_4. In contrast to salicylate and DNP, T_4 displacement is from TBG. At low levels of TCT, the T_4 that is not bound to TBG is found to be associated with TBPA. At very high levels of TCT there is also inhibition of binding of T_4 to TBPA, and the thyroxine thus displaced appears on albumin (Table I).

In view of the potent T_4 displacing action of TCT, it was important to establish that this com-

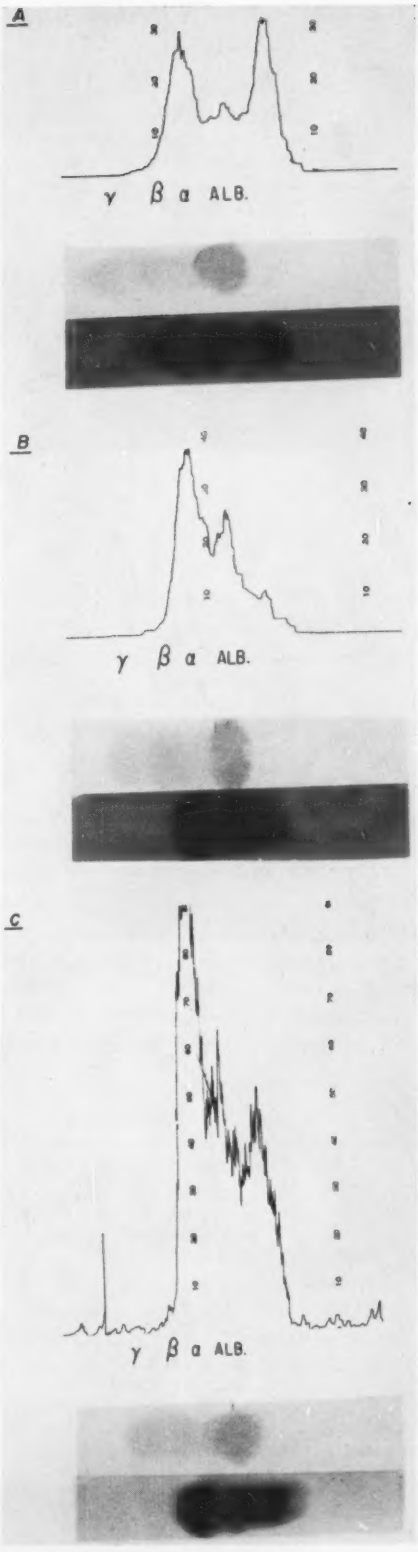
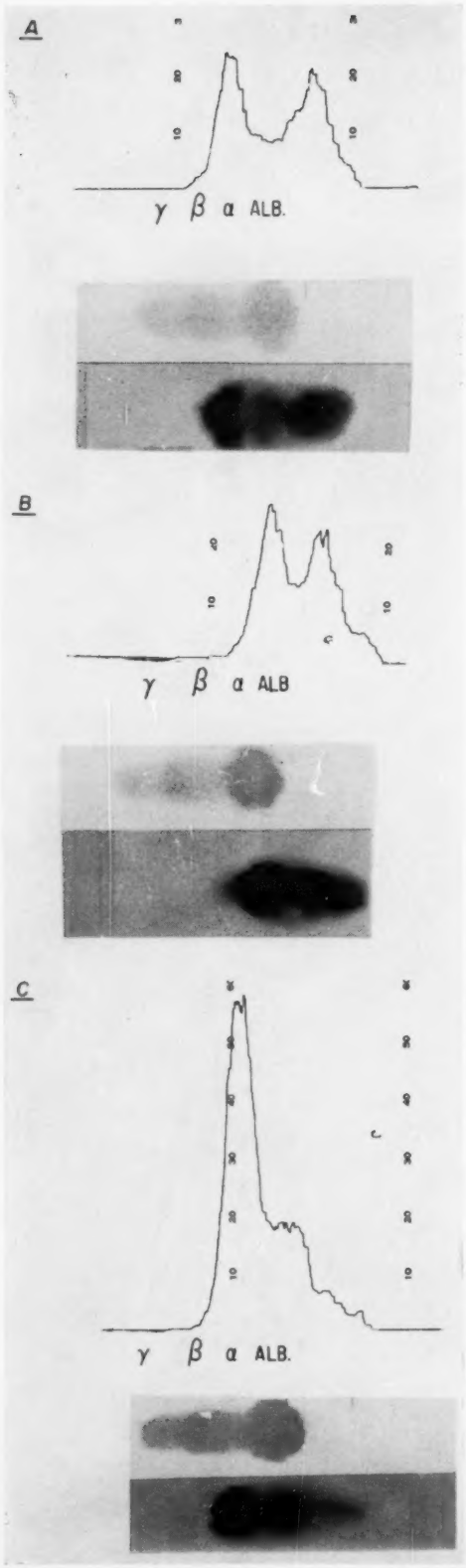


FIG. 2. THE EFFECT OF SALICYLATE ON THE ELECTROPHORETIC DISTRIBUTION OF RADIOACTIVE THYROXINE (0.28 μG PER ML OR 0.36 μM) IN HUMAN SERUM. A) Control serum; B) 3×10^{-3}M salicylate in the buffer; C) 3×10^{-3}M salicylate added to serum.



pound could, in fact, lower the PBI when administered to the intact animal since previous observations were not unequivocal (3, 4). Although we were able to demonstrate such an effect in rats, the results with guinea pigs were more dramatic and are listed in Table II. A 30 to 35 per cent depression in the PBI could be demonstrated, albeit at rather large doses of TCT. Since this T_4 analog does have very weak thyromimetic activity, it seemed important to rule out an indirect effect of TCT, e.g., via the pituitary TSH mechanism. For this reason thyroidectomized guinea pigs on constant doses of T_4 were utilized. It can be seen that under these conditions TCT lowered the PBI by nearly 50 per cent despite the very high initial PBI level. A similar PBI depression in athyreotic subjects receiving replacement therapy has been reported after DNP treatment in man (13) and after salicylate treatment in rats (14).

Oppenheimer, Fisher, Nelson and Jailer have recently shown that patients chronically treated with diphenylhydantoin show depressed values of PBI and have suggested that this might have resulted from T_4 displacement from one of the thyroxine-binding proteins (5). This proved to be the case (see Table I), and at drug concentrations that obtain clinically (15) there was marked displacement of T_4 from TBG to prealbumin. It may also be noted that in the presence of a constant amount of diphenylhydantoin, increasing concentrations of thyroxine result in increasing amounts of T_4 bound to TBG. With 10^{-4} M diphenylhydantoin, the amount of T_4 bound to TBG increased from 0.03 μg per ml at T_4 concentration of 0.07 μg per ml to 0.065 μg per ml at a T_4 concentration of 0.28 μg per ml, and at the highest T_4 concentration to 0.15 μg per ml. This would be expected when two compounds compete for the same site. Similar considerations apply to the other compounds studied. Even at the highest level of diphenylhydantoin (we were limited by the solubility at this pH) there was no displacement from prealbumin, in marked contrast to the results obtained with TCT (Table I). This may

FIG. 3. COMPARISON OF THE DISPLACEMENT OF RADIOACTIVE THYROXINE (0.15 μG PER ML OR 0.27 μM) FROM HUMAN SERUM PROTEINS PRODUCED BY DL-TETRACHLOROTHYRONINE AND SALICYLATE. A) Control serum; B) 3×10^{-4}M tetrachlorothyronine in the buffer; C) 3×10^{-3}M salicylate in the buffer.

TABLE I

The distribution (in per cent) of thyroxine on serum protein fractions as a function of the concentration of various displacing agents

Agent (molarity in buffer)	0.15 μg T_4/ml (0.19 μM)			0.28 μg T_4/ml (0.36 μM)			1.54 μg T_4/ml (2.0 μM)			2.08 μg T_4/ml (2.7 μM)		
	TBG	A	PA[12]†	TBG	A	PA[11]	TBG	A	PA[5]	TBG	A	PA[5]
Control ±SD	38 ± 2.4	20 ± 1.2	43 ± 1.1	38 ± 4.6	25 ± 2.2	37 ± 5.7	13 ± 1.0	32 ± 1.3	55 ± 1.7	15 ± 1.2	37 ± 8.6	48 ± 9.4
Salicylate												
3×10^{-4}				52	24	24				18	35	47
1×10^{-3}				61	27	12				21	59	20
3×10^{-3}	61	30	9	71	25	5						
2,4-Dinitrophenol												
1×10^{-5}	42	17	42									
3×10^{-5}				39	25	36				13	39	48
1×10^{-4}	55	32	13	56	28	16				11	54	35
3×10^{-4}	63	32	5				15	74	11			
Tetrachlorothyronine												
1×10^{-7}	32	27	41									
3×10^{-7}	20	36	44	18	41	41						
1×10^{-6}	11	48	42	14	45	41						
3×10^{-6}	6*	47*	48*	5	57	38						
1×10^{-5}	4	69	26				4	77	19			
5,5-Diphenylhydantoin												
3×10^{-5}				29	30	41						
1×10^{-4}	21	30	49	24	29	47	10	31	59			
3×10^{-4}	14	27	59	15	30	55						
6×10^{-4}	11	29	60				10	33	54			

* T_4 concentration for this experiment of 0.19 μg per ml (0.24 μM).
† Number of determinations at each level in brackets.

imply a difference at the prealbumin binding site, although higher concentrations of the drug might have shown displacement here also. In any case, the present finding, that diphenylhydantoin competes with thyroxine for sites on TBG is a reasonable explanation for the depression of PBI. It is surprising, however, that a compound so unlike the iodothyronines can compete for what appears to be a highly specific binding site on TBG.

DISCUSSION

There is available a body of evidence which suggests that both the effect and the rate of degradation of thyroxine are functions of the concentration of unbound thyroxine (7). Any factor which decreases protein binding of T_4 will, if the total concentration of thyroxine is unchanged, increase the concentration of free thyroxine. Consequently, an important factor in the control of the PBI is the serum concentration of available binding sites for the T_4. Given a constant output of T_4 from the thyroid, loss of these sites by competition with a variety of drugs leads inevitably to a depression in the PBI level. This cause for the reduction of the PBI concentration in the circulation must be clearly differentiated from that resulting from a

TABLE II

The effect of tetrachlorothyronine on the PBI of guinea pigs

Experiment	Treatment	No. of guinea pigs	PBI	p*
I	Control	5	3.0 ± 0.34	
	TCT 0.6 mg/100 g/day for 5 days†	6	2.0 ± 0.33	<0.001
II	Control	6	2.6 ± 0.28	
	TCT 0.4 mg/100 g/day for 1.5 days†	6	1.7 ± 0.16	<0.001
III	L-T_4 6 μg/100 g/day‡	6	14.7 ± 1.0	
(All guinea pigs	L-T_4 6 μg/100 g/day‡	5	7.6 ± 0.86	≪0.001
thyroidectomized)	TCT 1.0 mg/100 g/day‡			
	Control	4	1.1 ± 0.29	

* p Values are with respect to the control groups in Experiments I and II and with respect to the T_4-injected group in Experiment III.
† In two divided doses per day.
‡ The injection schedule is listed in Methods.

decrease or absence in the thyroxine-binding proteins, as in the case of nephrosis (16), congenital absence of a binding protein (17), or after treatment with methyl testosterone (18). Presumably the homeostatic mechanisms operating to reduce the PBI are similar in all these circumstances.

Inhibition of thyroxine binding to prealbumin has been clearly shown with both salicylate and dinitrophenol. Since there is no evidence that, in the concentrations used, either drug denatures serum proteins, and since there is a good correlation between concentration of drug and degree of inhibition of thyroxine binding, it may be assumed that these drugs compete with thyroxine for binding sites on TBPA. It would appear from the quantities of drug required that the association constant of both of these drugs for TBPA is several orders of magnitude lower than that of thyroxine. Neither of these drugs affects T_4 binding to TBG.

It is probable that addition of the drug to the buffer is the better method. If the association constant of drug for TBPA is not high it seems quite likely that if drug is added only to serum, there will be, during electrophoresis, a continual removal of unbound drug. If drug is added to the buffer, a reservoir is provided so that the concentration of drug in the buffer will approach the concentration of free drug. As a result of the uncertainty in the concentration of drug bound to protein, it is not possible to calculate accurately a ratio of binding constants of drug/T_4 for TBPA. Doubt has recently been expressed regarding the physiological importance of TBPA as a binding protein for T_4 at pH 7.6 (19). The present data with salicylate and DNP are nevertheless compatible with a significant role for TBPA.

The results with TCT are more easily interpreted because of the apparent high affinity constant of this compound for TBG; although bound less strongly, its binding affinity for TBG probably differs from that of T_4 by less than an order of magnitude. Since equilibrium is not attained in electrophoresis, a more exact statement of the relative affinities is not possible. At high concentrations TCT also inhibits T_4 binding to TBPA. Diphenylhydantoin appears not to have this second action. None of the compounds tested appeared to inhibit T_4 binding to albumin. This may be a quantitative phenomenon, since albumin is present in serum at a concentration of $\sim 5 \times 10^{-4}$ M [with four sites for T_4 (20)] and extremely high concentration of the drugs would be required. Furthermore, it is unlikely that T_4 binding to albumin normally plays a significant role in thyroxine metabolism, since the PBI of analbuminemic patients is normal (21). It is highly probable that other drugs will be found that can alter the PBI by this mechanism. It would behoove investigators studying thyroid function during therapy with presumably unrelated drugs to take cognizance of this fact.

It may be concluded, therefore, that a decrease in the number of available binding sites either in TBG or in prealbumin, produced by TCT and diphenylhydantoin or by DNP and salicylate, will (by mass law relations) increase the free T_4 at the expense of the fraction bound. This will in turn accelerate T_4 disappearance from the circulation and ultimately lower the PBI to a new steady state value at which the free T_4 concentration may be perfectly normal. The *in vivo* experiments with TCT, which produced a consistent fall in the PBI both in normal guinea pigs and in thyroidectomized, thyroxine-treated animals, offer only indirect support for this hypothesis, since no electrophoretic results on guinea pig sera are reported. Studies of guinea pig sera by the above techniques showed T_4 binding only in the albumin area, and T_4 displacement from one protein to another within this area would not be detectable.

The above considerations may also be applicable in certain short-term assays of thyromimetic (and antithyroxine) materials. Are such effects, as for example, goiter prevention, due to inherent thyroxine-like activity or do they merely represent an elevation of free T_4 as a result of thyroxine displacement from one of the serum proteins?

Can decreased T_4 binding explain the other phenomena produced by DNP and salicylate? Of the diverse effects of salicylate on thyroid function, loss of binding sites can probably explain the accelerated disappearance of radioactive T_4 from the circulation (2, 22, 23). Whether or not the other effects can be similarly explained is not certain at present. The whole problem must be reconsidered from the point of view of intracellular binding proteins and the effect of the drugs upon these.

SUMMARY

The binding of thyroxine to human serum proteins has been studied by paper electrophoresis in $(NH_4)_2CO_3$ buffer at pH 8.4.

1. Salicylate and 2,4-dinitrophenol inhibit thyroxine binding to prealbumin, the displaced thyroxine appearing in the thyroxine binding α-globulin area.

2. Diphenylhydantoin inhibits thyroxine binding to the thyroxine-binding α-globulin, displacing it onto prealbumin.

3. DL-tetrachlorothyronine displaces thyroxine from the thyroxine-binding α-globulin, and at high drug concentrations also from prealbumin. Tetrachlorothyronine given *in vivo* lowers the level of serum protein-bound iodine in intact and in thyroidectomized, thyroxine-treated guinea pigs.

4. It is concluded that loss of binding sites on either of the major thyroxine-binding proteins can explain the lowering of the serum protein-bound iodine produced by the four drugs studied when they are given *in vivo*.

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EXTRACTION AND RELEASE OF INDIVIDUAL FREE FATTY ACIDS BY THE HEART AND FAT DEPOTS *

By MARTIN E. ROTHLIN † AND RICHARD J. BING

(From the Department of Medicine, Wayne State University College of Medicine, Detroit, Mich.)

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It has previously been demonstrated (1) that in the postabsorptive state energy production of the heart depends largely on fatty acid metabolism; both esterified and nonesterified or free fatty acids (FFA) are taken up by the heart muscle. Although the general turnover rate of individual FFA has been studied, no investigations have been undertaken to discover their fate in the heart muscle.

After administration of C^{14}-labeled palmitic, oleic or linoleic acid which had been bound to albumin *in vitro*, Fredrickson and Gordon (2) found no difference in fractional turnover rate of these individual FFA in the plasma of the intact subject. General conclusions from these observations are difficult, since Bates and Olson (3) have pointed out that in the animal organism the fate of fatty acids bound to albumin *in vitro* may be different from that of endogenous FFA.

The present study deals with the myocardial extraction of individual FFA. Since, in the course of this investigation, it was found that the rate of myocardial uptake of individual FFA differed, the work was extended to include the release of individual FFA by adipose tissue.

METHODS

The patients studied in this report were free of metabolic disorders, and none of them was in congestive heart failure. Food was withheld for 12 hours prior to the test. For the animal studies, mongrel dogs were used from which food was withheld for at least 16 hours prior to the experiment. The dogs were anesthetized with pentobarbital (30 mg per kg intravenously). Coronary venous blood of man and dog was obtained by catheterization of the coronary sinus (4). Simultaneously, arterial blood was collected through an indwelling needle from the brachial artery (man) or from the femoral artery (dog). Blood from the saphenous vein (dog), which drains largely subcutaneous adipose tissue, was collected from a needle inserted into this vessel.

Free fatty acids were determined with the technique of Gordon, Cherkes and Gates (5). For gas chromatography, the FFA were extracted with 90 per cent ethanol-water containing 0.2 per cent NaOH from the iso-octane extract prepared during this procedure. After removing the contaminating esters from this alkaline ethanol-water phase by petroleum ether extraction, the ethanol-water phase was acidified and the FFA extracted with light petroleum. The solvent was then evaporated and the FFA converted into methyl esters according to the method used by Hallgren, Stenhagen, Svanborg and Svennerholm (6). Analytical separation was carried out on a Barber Colman gas chromatograph, model 10, provided with an argon ionization detector (7). Ethylene glycol succinate polyester was used as stationary phase at a temperature of 179° or 182° C (8). FFA peaks were identified by comparison of their retention times with those of known standard fatty acids. Peak areas were measured by triangulation and by electronic integration. On each sample, three chromatograms were performed and evaluated by both methods. Since small arteriovenous differences of the FFA composition were involved in these observations, the reproducibility of the method had to be ascertained. Ten successive gas chromatographic analyses of a standard mixture of known composition were therefore performed. As in the remainder of the experiments, peak areas were determined both by triangulation and electronic integration. Table I represents the mean value, maximum deviations and variation coefficient for each component. It may be seen that the variation coefficients of the recoveries are below 1 per cent with the exception of palmitoleate. Table II illustrates the reproducibility of the applied method. Four aliquots of the same dog plasma were analyzed for their FFA composition. On each sample, as in the remainder of the experiments, three chromatograms were performed. The determinations were all within 0.7 per cent.

According to Lovelock (7), the mass sensitivity of the ionization detector (i.e., the response of the detector to a given mass of substance) is constant, within the

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† Fellow of the Foundation for Biol.-Med. Research (Switzerland).

TABLE I

Fatty acid composition of a standard mixture analyzed by ten gas chromatograms

	Palmitate	Palmitoleate	Stearate	Oleate	Linoleate
	%	%	%	%	%
Weighed	25.2	7.8	19.8	28.6	18.6
Mean of recoveries	27.8	7.1	18.0	29.7	17.4
Range of recoveries	27.7–28.2	6.9–7.2	17.8–18.1	29.6–30.0	17.1–17.8
Variation coefficient of recoveries	0.96	1.67	0.59	0.53	0.71

limits of precision of the method, for the methyl esters of the higher fatty acids. The FFA composition, analyzed by gas chromatography, is therefore expressed in per cent by weight of the sum of the methyl esters of palmitic, palmitoleic, stearic, oleic and linoleic acids (Tables I, II, III, IV and V). According to Dole and co-workers (9), these fatty acids are the main components of FFA and comprise about 85 per cent of this plasma lipid fraction in man. Several fatty acids of a chain length of 6 to 14 C-atoms, as well as higher unsaturated fatty acids with 18 and 20 C-atoms, contribute the remaining 15 per cent to the total FFA; these were not determined. Since, therefore, only 85 per cent of the plasma FFA was analyzed, these calculated values are too high and must be corrected to afford a comparison with other published data. Consequently, Table III contains not only the experimentally determined values but also the mean values calculated on the basis that only 85 per cent of the total FFA was determined.

Total FFA levels represent the amount of FFA in plasma expressed in milliequivalents per 100 ml. Individual FFA levels were calculated from total FFA levels and the composition of FFA. The percentage by weight in which the composition of individual FFA is given was transformed into moles per cent. Eighty-five per cent of the total FFA levels determined by titration was used as the sum of the combined individual FFA levels. Individual FFA levels were calculated as follows: $0.85 \times$ total FFA (mEq/100 ml plasma) $\times$ concentration of individual FFA (mole fraction). The percentage myocardial extraction of total or individual FFA was calculated as follows: $(a.FFA - c.s.FFA) \times 100/a.FFA$, where a.FFA represents total or individual FFA level in milliequivalents per 100 ml arterial plasma, and c.s.FFA represents total or individual FFA level in milliequivalents per 100 ml coronary sinus plasma. The percentage release of total or individual FFA by adipose tissue was calculated with the formula: $(s.v.FFA - a.FFA) \times 100/s.v.FFA$, where s.v.FFA represents total or individual FFA level in milliequivalents per 100 ml of saphenous vein plasma, and a.FFA represents total or individual FFA level in milliequivalents per 100 ml arterial plasma. As outlined above, it was assumed in these calculations that the FFA not determined comprise the same percentage in arterial and coronary sinus blood (15 per cent). It must be considered that the proportion of these components in coronary sinus blood could differ from that in arterial blood. This, however, would not affect the observation that the percentage extraction or the percentage release of an individual FFA is different from that of another, but it would affect the absolute estimates of extraction and release of individual FFA.

RESULTS

Myocardial extraction of individual FFA. Table III illustrates the arterial and coronary sinus levels of total FFA in fasting patients. The mean arterial FFA level found was 0.0692 mEq per 100 ml with a range of from 0.0368 to 0.1227 mEq per 100 ml plasma. The mean FFA level in coronary sinus blood was 0.0509 mEq per 100 ml plasma with a range of from 0.0281 to 0.0931 mEq per 100 ml plasma. Table IV illustrates the results obtained in dogs. The mean arterial FFA level was 0.0328 and that in the coronary sinus 0.0155. The difference between values obtained in patients and in dogs may have been the result of pentobarbital anesthesia used in the animals (10).

Data on the composition of FFA in arterial and coronary sinus blood are illustrated in Tables III and IV. Oleic acid was the largest component in man and in dog (50.0 and 44.2 per cent, respec-

TABLE II

Gas chromatographic analysis of the FFA composition of four samples of the same dog plasma

Sample no.	FFA composition*				
	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid
	%	%	%	%	%
1	27.5	3.0	15.1	41.4	13.0
2	27.2	2.9	15.0	41.8	13.1
3	27.7	2.9	15.7	41.2	12.5
4	27.8	2.9	15.6	41.3	12.4

* Expressed in per cent by weight of the sum of the methyl esters of the 5 determined fatty acids.

TABLE III

Coronary arteriovenous difference of FFA level and composition in man

Patient no.		FFA levels	FFA composition*				
			Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid
		mEq/100 ml plasma	%	%	%	%	%
1	A†	0.0550	28.6	2.7	10.0	48.0	10.7
	CS†	0.0414	30.0	2.7	10.4	45.4	11.5
2	A	0.0519	28.5	2.7	10.2	46.8	11.8
	CS	0.0403	33.0	2.4	10.7	42.3	11.7
3	A	0.0582	26.1	2.7	11.4	52.0	7.8
	CS	0.0426	34.0	4.6	14.0	39.8	7.6
4	A	0.0764	28.7	4.0	8.9	52.5	5.9
	CS	0.0561	31.6	3.6	9.5	49.4	5.9
5	A	0.1227	26.9	3.8	5.9	52.9	10.5
	CS	0.0931	28.1	3.8	6.1	50.8	11.2
6	A	0.0368	30.0	3.5	11.6	47.5	7.4
	CS	0.0281	33.7	3.7	10.4	44.7	7.5
7	A	0.0834	29.2	2.8	8.4	50.6	9.0
	CS	0.0545	31.7	2.9	9.7	46.2	9.5
Mean	A	0.0692	28.3	3.2	9.5	50.0	9.0
			24.0‡	2.7	8.1	42.5	7.7
	CS	0.0509	31.7	3.4	10.1	45.5	9.3
			26.9‡	2.9	8.6	38.7	7.9

* Expressed in per cent by weight of the sum of the methyl esters of the 5 determined fatty acids.
† A = arterial; CS = coronary sinus.
‡ Corrected values; see text under Methods.

TABLE IV

Coronary arteriovenous difference of FFA level and composition in dog

Dog no.		FFA levels	FFA composition*				
			Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid
		mEq/100 ml plasma	%	%	%	%	%
1	A	0.0241	28.7	3.8	15.7	39.6	12.2
	CS	0.0087	31.9	3.4	22.2	27.7	15.1
2	A	0.0530	22.7	5.2	9.5	53.9	8.8
	CS	0.0276	26.4	4.7	13.0	45.1	10.7
2	A	0.0476	26.3	3.0	10.8	48.5	11.4
	CS	0.0169	30.8	2.6	15.6	37.6	13.3
4	A	0.0239	29.4	3.4	14.8	44.0	8.4
	CS	0.0097	36.6	3.7	17.4	34.2	8.1
5	A	0.0280	35.6	2.3	14.6	34.9	12.6
	CS	0.0179	39.2	2.1	15.9	31.0	11.8
6	A	0.0203	29.8	4.0	13.6	44.0	8.6
	CS	0.0125	34.9	4.3	15.9	36.3	8.6
Mean	A	0.0328	28.8	3.6	13.2	44.2	10.3
	CS	0.0155	33.3	3.5	16.7	35.3	11.3

* Expressed in per cent by weight of the sum of the methyl esters of the 5 determined fatty acids. Abbreviations are as in Table III.

tively, in arterial blood). The second highest was palmitic acid (28.3 per cent in man, 28.8 per cent in dog). The corrected figures in Table III for arterial palmitic and palmitoleic acids were in good agreement with those reported by Dole and his associates (9). However, the fractions of stearic and linoleic acids were lower, while the proportion of oleic acid was higher in the present series.

Tables III and IV illustrate the differences between the FFA composition of arterial and coronary sinus blood. In man (Table III) the percentage of palmitic acid in coronary sinus blood was higher than that in arterial blood, while the proportion of oleic acid in coronary sinus blood was lower than in arterial blood. Both of these differences are statistically significant ($p < 0.01$ and $p < 0.025$, respectively). Similar results were found in dogs with the exception that stearic acid was also significantly higher in coronary sinus than in arterial blood ($p < 0.01$) (Table IV).

These changes reflect a different rate of myocardial uptake of individual FFA. This is illustrated in Figure 1 in which the percentage myocardial extractions of individual FFA are outlined. Thus, the human heart extracted from arterial plasma 17.4 per cent of the free palmitic acid as compared with 32.9 per cent of the free oleic acid. The dog heart removed 37.1 per cent of the free stearic acid from arterial plasma, while 44.4 per cent of free palmitic acid and 61.1 per cent of the free oleic acid were extracted.

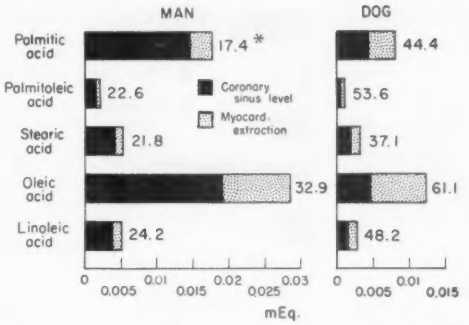

FIG. 1. MYOCARDIAL EXTRACTION OF INDIVIDUAL FFA IN MAN AND DOG. The total length of the bars represents the arterial, and the dark portion the coronary sinus level of individual FFA, expressed in mEq per 100 ml plasma. The stippled portion of the bars corresponds to the myocardial extraction. Each figure * represents the percentage extraction of an individual FFA. (Mean values obtained from seven subjects and six dogs, respectively.)

Release of individual FFA from adipose tissue. Table V illustrates the release of individual FFA by adipose tissue of the dog. The mean total FFA level in arterial plasma was 0.0322 mEq per 100 ml and that of saphenous venous plasma 0.0454 mEq per 100 ml, illustrating release of FFA from the fat depots. Table V also shows that there were differences in release of individual fatty acids by the adipose tissue. For example, the proportion of oleic acid to total FFA was higher in saphenous vein blood than in arterial blood, while the reverse was the case for palmitic and stearic

TABLE V

Saphenous arteriovenous difference of FFA level and composition in dog

Dog no.		FFA levels	FFA composition*				
			Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid
		mEq/100 ml plasma	%	%	%	%	%
7	A†	0.0370	31.9	3.2	12.1	38.3	14.5
	SV†	0.0570	29.0	4.4	10.8	42.2	13.6
8	A	0.0288	27.6	2.0	19.8	35.7	14.9
	SV	0.0410	27.1	2.8	17.2	39.2	13.7
9	A	0.0473	24.7	4.5	9.7	52.5	8.6
	SV	0.0635	22.8	5.4	8.1	55.0	8.6
10	A	0.0157	33.7	3.3	17.0	38.4	7.6
	SV	0.0199	28.0	3.7	13.6	46.3	8.4
Mean	A	0.0322	29.5	3.2	14.7	41.2	11.4
	SV	0.0454	26.7	4.0	12.5	45.7	11.1

* Expressed in per cent by weight of the sum of the methyl esters of the 5 determined fatty acids.
† A = arterial; SV = saphenous vein.

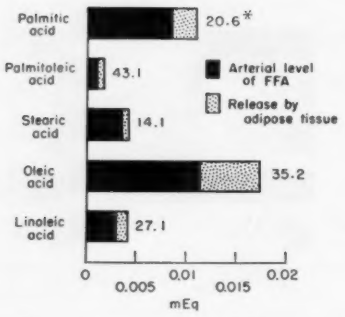

FIG. 2. RELEASE OF INDIVIDUAL FFA BY SUBCUTANEOUS ADIPOSE TISSUE IN DOG. The total length of the bars represents the saphenous venous, and the dark portion the arterial level of individual FFA, expressed in mEq per 100 ml plasma. The stippled portion of the bars corresponds to the release by adipose tissue. Each figure * represents the percentage release of an individual FFA. (Mean values obtained from four dogs.)

acids. The release of individual FFA by fat depots is graphically illustrated in Figure 2. It is seen that 43.1 per cent of the free palmitoleic acid and 35.2 per cent of the free oleic acid present in saphenous vein plasma were released by adipose tissue; only 20.6 and 14.1 per cent of the free palmitic and stearic acids, respectively, were released.

DISCUSSION

The values for total FFA in plasma agree with those published by Gordon and his associates (5) and with those previously obtained in this laboratory (1). A comparison of the composition of the FFA fractions obtained in this series with that found by Dole shows similar values for palmitic and palmitoleic acids. However, in the present series, the fractions of stearic and linoleic acids are lower, while the proportion of oleic acid is higher. This may well be due to differences in subjects on whom these tests were performed. In the series of Dole and co-workers (9), determinations were made on fasting male medical students and on laboratory personnel. His subjects were healthy and of good dietary habits. In this series, although all patients were fasting for at least 12 hours, the subjects were chosen at random from the patients of a large municipal hospital. However, none of the patients studied in this series had any known metabolic disease.

As illustrated in Tables III and IV, the heart extracts considerable amounts of FFA. This is in line with previous publications from this laboratory (1) as well as with the data of Gordon and his associates (5) and of Olson (11). As illustrated in Tables III and IV, significant differences exist between the FFA composition of arterial and coronary sinus blood. The proportion of oleic acid in coronary sinus blood is lower than that in arterial blood, demonstrating a high degree of extraction (Figure 1). In both man and dog, the percentage of palmitic acid in coronary sinus blood is higher than that in arterial blood, and in the dog the proportion of stearic acid is also higher in coronary sinus blood, demonstrating a low rate of myocardial uptake. Thus, although the percentage of an individual FFA may be greater in coronary sinus than in arterial blood, the level of this FFA can still be lower as a result of its myocardial extraction (Figure 1).

These observations illustrate that the human as well as the dog heart extracts all individual FFA analyzed. However, there exist significant differences in the rate of myocardial uptake of individual FFA. This is indicated by the relatively larger percentage extraction of free oleic acid. Oleic acid is also released to a larger extent by subcutaneous adipose tissue. The percentage myocardial extraction of free palmitic and stearic acids as well as their release from adipose tissue is low (Figure 2). These observations suggest different turnover rates of individual FFA. However, definitive proof of this is lacking, since release of free fatty acids and their uptake has been investigated from two sites only, the subcutaneous fat and the heart.

Several possible explanations exist for the different rates of myocardial uptake of individual FFA as estimated by their coronary arteriovenous balances. In the first place, a preferential uptake of oleic acid by the heart muscle cell itself is a possibility. However, observations by Neptune, Sudduth, Foreman and Fash (12), obtained on striated muscle incubated in a medium containing different C^{14}-labeled FFA, make this unlikely. These investigators measured the uptake and oxidation of free oleic acid and found it not to be higher than that of other FFA. Conversion of oleic acid to stearic acid by hydration must also be considered. This is unlikely since it has been shown that hydration of oleic acid appears not to

take place in the animal organism (13). Since the amount of adipose tissue drained by the coronary sinus is much lower compared with that of heart muscle tissue, it is unlikely that FFA release by myocardial fat tissue influences markedly the FFA composition of coronary sinus blood.

It should be considered that the myocardial uptake of esterified fatty acids, which exceeds the myocardial extraction of FFA in the postabsorptive state (1), might influence the coronary arteriovenous balance of individual FFA. Plasma triglycerides play an important role in the fatty acid transport of the intact organism. They may enter the cells, as in the case of adipose tissue (14), after previous hydrolysis, apparently catalyzed by lipoprotein lipase (15), or they are taken up as triglycerides, as in the case of the liver (16, 17), which contains no lipoprotein lipase. High lipoprotein lipase activity has been demonstrated in rat heart muscle (18), and it appears, therefore, not unlikely that hydrolysis of plasma triglycerides by lipoprotein lipase precedes their uptake by the myocardium. Borgström (19) has demonstrated *in vitro* an exchange between plasma triglyceride fatty acids and FFA in the presence of lipoprotein lipase. In line with this concept are observations of Havel and Fredrickson (20) who showed in the intact dog that C^{14}-labeled triglyceride fatty acids, when injected intravenously, reappear in the plasma FFA fraction. Thus, the FFA in coronary sinus blood could partly originate from hydrolysis of plasma triglycerides through the action of lipoprotein lipase and may therefore have a different composition from the FFA in arterial blood. Finally, an exchange of FFA with other esterified fatty acids, such as phospholipid fatty acids, might also account for some of the coronary arteriovenous difference of the FFA composition.

SUMMARY

The myocardial extraction of individual free fatty acids (FFA) and their release by subcutaneous adipose tissue was investigated in man and dog.

In the postabsorptive state, the human as well as the dog heart extracted a consistently higher percentage of free oleic acid than of any other FFA present in arterial blood. This acid was also released at a higher rate by fat depots of the dog. These findings suggest a high turnover rate of free oleic acid.

Several possibilities were examined to explain the high myocardial extraction of free oleic acid. It appears likely that an exchange between plasma triglycerides and free fatty acids in heart muscle, resulting from lipoprotein lipase activity, may be responsible.

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Correction Notice

The values listed for carbon dioxide content of the anemic subjects in the article by Sproule, Mitchell and Miller, "Cardiopulmonary Physiological Responses to Heavy Exercise in Patients with Anemia," which appeared in the February issue of this JOURNAL, 1960, **39**, 378, were determinations of whole blood carbon dioxide content. Values listed for the carbon dioxide content of normal individuals, which appeared in the same article, were determinations of plasma carbon dioxide content.

ARTERIOVENOUS DIFFERENCES OF THE NOREPINEPRINE–LIKE MATERIAL FROM NORMAL PLASMA AND INFUSED NOREPINEPHRINE *

By JOHN M. WALLACE,† HUGO GARCIA,‡ AND EUGENE A. STEAD, JR.

(From the Department of Medicine, Duke University Medical Center and the Veterans Administration Hospital, Durham, N. C.)

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All physiological stimuli result in changes in the level of activity of the autonomic nervous system. A method of monitoring the over-all activity of the adrenergic nerves and adrenal medullae would greatly simplify the interpretation of many physiological events. The present work began with the assumption that blood levels of norepinephrine (NE) and epinephrine (E) might reflect adrenergic activity. Therefore, our initial objective was to modify one of the existing chemical methods for the measurement of NE and E so that it would be suitable for our purposes. However, we became impressed with the controversy in the literature over the chemical identification of plasma NE and E and, as a result, adopted the dual criteria that plasma material must have not only the chemical properties of NE and E but must be removed by the tissues like NE and E to justify identification as these substances.

The trihydroxyindole (THI) method of Lund (1–4) and the ethylenediamine (EDA) method of Weil-Malherbe and Bone (5–7) are the two methods currently considered suitable for analysis of plasma NE and E. Neither method is specific. In this respect, the EDA method is inferior to the THI method (6, 8, 9), a fact which is emphasized by the false high readings of the former in uremia (10). However, the THI method may measure dopamine (3, 4), dihydroxyphenylalanine (2–4), isopropylnorepinephrine (2–4, 9), serotonin (2) or aureomycin (11), depending on the modification used. To a greater (EDA method) or lesser degree (THI method), the proponents of both methods have depended upon the fact that none of the possibly interfering analogs of NE and E has as yet been demonstrated in normal blood by fluorometry, paper chromatography or bioassay. Cohen and Goldenberg (3) cited the results of infusion experiments as important evidence that their method (THI) did not measure the metabolites of NE or E. Steady infusions produced steady blood levels, which would not be expected if accumulating metabolites were also determined. We have had a similar experience with the EDA method.

The difficulty in preparing reagent blanks which fluoresce with the same intensity each day indicates that contaminants are capable of producing fluorescence which may be interpreted as NE and E. Both methods are subject to this difficulty. In our opinion, this type of nonspecificity has interfered more with the quantitative aspects of the methods than has nonspecificity due to possible interference by the metabolites of NE and E or other closely related compounds.

Whether or not either method as reported to date can accurately measure known NE and E in the amounts said to be present in normal plasma is also open to question. Price and Price (2), using the THI method, reported normal venous levels of 0.01 μg E per L and 0.34 μg NE per L. They used 15-ml samples of plasma but gave no data on their ability to distinguish unmodified plasma from plasma with added E and NE in amounts of 0.00015 μg E per 15 ml or of 0.0051 μg NE per 15 ml. They stated that differences in replicates of a single sample varied as much as did recoveries of small amounts of added material. They noted that their faded blanks varied in proportion to the amounts of E or NE which had gone into making them up. Cohen and Golden-

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TABLE I

Mean galvanometer deflections per 0.001 μg of NE or E added to 5-ml aliquots of plasma *

	Blue-green fluorescence	Yellow fluorescence
NE	5.7 (SD ± 1.1)	3.8 (SD ± 0.5)
E	1.6 (SD ± 1.0)	12.0 (SD ± 1.9)

* The actual measurements from which the table is derived were made on 9 different plasmas. Epinephrine in 2 concentrations (0.002 μg per 5 ml and 0.004 μg per 5 ml) and norepinephrine in 2 concentrations (0.004 μg per 5 ml and 0.008 μg per 5 ml) were determined in duplicate in each plasma.

berg (3) also used 15-ml plasma samples and reported normal levels of 0.06 μg E per L and 0.30 μg NE per L. They also gave no data on the difference between unmodified plasma and plasma with added E and NE in amounts of 0.0009 μg E per 15 ml or of 0.0045 μg NE per ml. They stated that "the small amounts of fluorescence above blanks detected in normal plasma specimens had associated with them a low degree of precision and reliability."

Vendsalu (4), using the THI method with samples of 10 ml, found normal levels of 0.07 μg E per L and 0.35 μg NE per L. He reported a recovery of 83 per cent of E and NE added to plasma in amounts which were, on the average, higher than these concentrations. In discussing normal plasma values, he remarked that the limit of sensitivity was governed by the blank and that resolution was diminished when the height of the sample peak was low and near to the blank. These problems have been given little attention in recent EDA papers (6–8, 12, 13).

None of the authors referred to above explains why he uses standard solutions of E and NE which are many times more concentrated than normal plasma. In our experience, instrument settings based on such standards are too insensitive for quantitative work with NE or E added to plasma in apparently normal concentrations.

We elected to explore further the potentialities of the EDA method because of greater blank stability at high sensitivities (14) and because the final extraction into isobutanol afforded the opportunity of effecting an increased concentration of the desired materials over the blank.

This paper will present data on four points: *1*) modifications of the EDA method which make it more quantitative at low concentrations of NE and E; *2*) the concentration of material in normal plasma with the fluorescent characteristics of NE and E; *3*) arteriovenous (A-V) differences of the material found in normal plasma which was assumed to be NE; and *4*) A-V differences of infused NE.

METHOD

The method of Weil-Malherbe and Bone (5), as modified by Aronow and Howard (15) and further modified by ourselves, is used. The blanks and samples are made stable and reproducible by scrupulous attention to sources of contamination, such as the glassware, ground-glass stoppers, room dust, tobacco smoke, impurities in reagents, and so forth. The volume of isobutyl alcohol used in the final extraction is 0.5 ml. This is read in a 6 × 6 mm square Pyrex cuvet in a Farrand model A fluorometer. The exciting light is the 436 mμ mercury line, and fluorescence is read at 485 mμ and above 530 mμ. The fluorometer-galvanometer unit is set at the same sensitivity daily by means of fluorescent filter glass. The sensitivity selected was that which gave readings for normal plasma near the center of the galvanometer. Standard solutions read near the top of the galvanometer at this setting.

Heparin was found to be a source of variability and was replaced by ethylenediamine tetraacetic acid (EDTA) as the anticoagulant. Blood samples cooled in ice water are stable for at least 90 minutes. Thiosulfate preservative was extensively investigated and found to be unnecessary. All samples, blanks and standards are run in duplicate. Five-ml samples of plasma and 125 mg alumina are used.

RESULTS

A. Sensitivity and accuracy. Standards containing 0.002 and 0.004 μg of E and 0.004 and 0.008 μg of NE per 5 ml of plasma were used to determine the number of galvanometer deflections given by NE and E with the two sets of filters. The reading of 485 mμ (blue-green filter) indicates primarily NE and that above 530 mμ (yellow filter) indicates primarily E. The average value given by the standards over a period of days was used in calculating the NE and E content of plasmas containing unknown amounts of NE and E. This allowed us to calculate the deviation of the standard from the mean (Table I). As the concentrations of NE and E in the standards and unknowns were of the same order of magnitude, losses in the procedure were equal in the two groups and the mean recovery in a series of known solutions approached 100 per cent (Table II). Changes in batches of reagents or a new

light source or photomultiplier tube in the fluorometer affected the number of galvanometer deflections per unit of material and required the establishment of new sets of values.

Table II gives the recovery of mixtures of standard amounts of NE and E. Comparison of these data with the normal values given below clearly shows that this method is capable of the quantitative recovery of added NE in the apparent normal range and that physiological concentrations of known materials can be used as the regular standards. The amount of material in normal plasma giving the fluorescent pattern of E is too small for accurate quantitation in a single determination. In a series of determinations, plasma with E added in this concentration can be separated from plasma without added E.

Standards similar to those reported in Table I can be run each day or each week. If the sensitivity of the fluorometer-galvanometer unit is kept constant by the use of the fluorescent glass filter, and if the reagent blanks are constant, weekly standards are sufficient.

B. Normal values for material in plasma measured as NE and E. The average arterial and venous concentrations of apparent NE and E found in normal university men are given in Table III. All samples were obtained through indwelling needles from subjects lying quietly in bed. These values are lower than those previously reported with the EDA method (5–10, 13, 16, 17). In our opinion, the values are lower than those usually reported for the EDA method because of the elimination of many fluorescent contaminants. The values for NE are 100 per cent higher than those reported with the THI method (2–4). A part of this difference is predictable, as our experimental design allowed approximately 100 per cent recovery of added material, and the THI method supposedly recovers about 80 per cent of added material. This accounts for 40 per cent of the difference. Data which allow detailed analysis of recoveries with the THI method at the levels of concentration under discussion are not available. This makes interpretation of the significance of the remainder of the difference uncertain.

TABLE II

Calculated mean amounts of NE and E added as a mixture to 5-ml aliquots of plasma on 9 different days *

Amounts of NE and E actually added to 5 ml plasma		Amounts of NE and E calculated to be in the mixture	
	µg		*µg*
NE	0.004	NE	0.0037 (SD ± 0.0005)
E	0.002	E	0.0018 (SD ± 0.0002)

* The measurements were made on the same days as those in Table I. The calculations are based on the observed galvanometer readings with the blue-green and yellow filters from the aliquots containing only NE or E (Table I). Because of the experimental design, it is assumed that recoveries would approach 100% if the series were expanded indefinitely.

TABLE III

Arterial and venous concentrations of the material measured as NE in normal subjects

	Mean	SD
	µg/5 ml plasma	
A. 47 Subjects, simultaneous arterial-femoral venous samples		
Artery*	0.0036	±0.0018
Vein	0.0040	±0.0015
B. 7 Subjects, 19 simultaneous arterial-antecubital venous samples		
Artery*	0.0037	±0.0017
Vein	0.0040	±0.0019

* The difference between arterial and venous concentrations is not significant, $p > 0.05$. The difference becomes significant when the 47 determinations in A are combined with the 19 determinations in B and analyzed together.

C. Is there an arteriovenous difference of the material measured as NE and E? Using a modified THI method, Vendsalu (4) reported that the mean A-V differences across an extremity in 29 healthy subjects were 0.16 µg E and − 0.09 µg NE per L. To be certain that our method was accurate enough to measure differences in these ranges, five sets of observations were made on five different days. Each day pooled plasma was divided into three aliquots. To one aliquot was added 0.00045 µg NE per 5 ml, to the second 0.0008 µg E per 5 ml, and nothing was added to the third. Thirty-four comparisons were made between the unmodified plasma and either of the other two aliquots. The technician working with the samples did not know the purpose of the experiment and did not know that half of the samples contained unmodified plasma. Twenty-four comparisons were made between plasma and plasma plus NE. There was enough spread in the readings so that the 12 highest plasma samples could not be distinguished from the 12 lowest

TABLE IV

*Arterial-antecubital venous differences of the material measured as NE and E in 7 subjects with and without occlusion of the wrist**

	Wrist unoccluded			Wrist occluded		
	Mean	SE	Significance of difference	Mean	SE	Significance of difference
	μg/5 ml plasma			*μg/5 ml plasma*		
NE	−0.0004	±0.0002	$p > 0.05$	−0.0004	±0.0003	$p > 0.10$
E	+0.0002	±0.00009	$p < 0.025$	+0.0002	±0.00007	$p < 0.005$

* The data were obtained from 19 A-V pairs with the wrist unoccluded and 19 pairs with the wrist occluded.

samples of plasma plus NE. All of the remaining 12 NE samples were higher than any of the remaining 12 plasma samples. Statistical analysis of all 24 comparisons showed the aliquots to be different ($p < 0.01$), the calculated mean differences having been 0.0007 ± 0.0006 μg NE per 5 ml. Actually, only 12 *random* comparisons of the two aliquots were necessary to demonstrate the difference (p 0.02). Although this method cannot *quantitate* differences in plasma concentration of 0.00045 μg NE per 5 ml, it does reliably identify such a difference in as few as 12 comparisons. It cannot identify this difference in a single comparison.

All ten samples of plasma plus E were higher than any of the ten plasma samples with which they were compared. The calculated mean difference was 0.0007 ± 0.0002 μg E per 5 ml. To our knowledge similar data have not been offered in support of the ability of any other method to measure differences in plasma concentrations of added NE and E in the range of apparent peripheral arteriovenous differences.

In view of the ability of this method to identify such differences in added NE and E in a relatively few comparisons, re-examination of the data in Table III is of interest. The small negative A-V difference of the material called NE (0.0004 μg per 5 ml plasma) observed across the leg in 47 subjects was not significant. Neither was the same difference noted across the arm in 19 samples from 7 subjects, the p value having been greater than 0.05 in both cases. If these data are combined, the A-V difference does become significant, p falling to less than 0.01. It appears that there is, on the average, a small negative A-V difference of the material called NE but that the arterial and venous concentrations are too similar in many people to permit its demonstration except in a sizable population.

The small positive A-V difference of the material called E (0.0002 μg per 5 ml) is significant. Interpretation of epinephrine data will not be included in this paper.

As noted above, some workers (2, 4), including ourselves at an earlier date (18), found higher levels of NE-like fluorescent material in venous blood than in arterial blood. It has been assumed that the venous material was true NE produced by the adrenergic nerves in the hand and forearm. In an attempt to clarify this point, indwelling needles were placed in the brachial artery and antecubital vein of seven normal men lying quietly in bed. Every 10 minutes, arterial and venous samples were drawn. For 3 minutes preceding each alternate drawing, a cuff at the wrist was inflated to 250 mm Hg. Data are given in Table IV. The mean A-V difference for the material called NE was negative, but not significantly so, and was unchanged by wrist occlusion. As was expected, venous blood in the samples collected with the wrist occluded contained less oxygen than did those without the occlusion.

These data are of interest because we expected the venous blood draining the hand to be a favorable site for the demonstration of NE. The arterial blood passing through A-V communications would have lost little NE in its passage through the tissues. The hand is well supplied with adrenergic nerves and, under the conditions of this experiment, continuous autonomic nervous activity is likely to be present. The addition of blood from areas where NE is made in the hand to a stream of venous blood from which no NE had been lost creates a situation which would appear favorable for the demonstration of the addition of NE to venous blood. However, the observed small negative A-V difference of the NE-like material (-0.0004 μg per 5 ml) was not significant

in the normal arm and was not further narrowed by exclusion of the hand from the circulation.

D. Do the endogenous material called NE and infused NE behave similarly in the blood stream? As pointed out above, no reported fluorometric method is entirely specific for NE and E. Both the THI and EDA methods are presumed to measure only NE and E in normal peripheral plasma. It appeared desirable to determine whether the material in plasma presumed to be NE was treated by the tissues in the same way as infused NE.

Nineteen normal subjects ranging in weight from 113 to 57 kg (mean, 80 kg) were given infusions of NE into an antecubital vein at the constant rate of 4 μg per minute (average, 0.05 μg per kg per minute). The average increase in blood pressure was 14/10 mm Hg and the average decrease in pulse rate was 8 beats per minute. Most subjects had no symptoms. Simultaneous blood samples from a brachial artery and a femoral vein were drawn before infusion and after at least 10 minutes of infusion. Table V summarizes the data. Concentration of the endogenous material resembling NE was greater in the artery in 8 subjects and greater in the vein in 11 subjects. The arterial and venous concentrations were not significantly different. The average concentration of the NE in arterial plasma produced by the infusion was twice the concentration of NE-like material present before infusion. Arterial levels from the infused material were higher than venous levels in all subjects, averaging nearly four times the femoral venous concentrations. In three subjects, no infused NE appeared in the femoral vein, removal in the leg having been complete. On the average, the leg removed 72 per cent of the arterial NE. In four subjects, antecubital venous samples (side opposite the infusion) were also measured and showed in each instance a positive A-V difference of infused NE. These data clearly demonstrate the efficient removal of arterial NE by the leg. By contrast, there was not a significant femoral A-V difference of the material measured as NE in resting plasma.

TABLE V

Femoral A-V differences of infused NE and the plasma material measured as NE in 19 subjects

	Plasma material measured as NE		Infused NE	
	Mean	SD	Mean	SD
	μg/5 ml plasma		*μg/5ml plasma*	
Artery	0.0039*	±0.0015	0.0081†	±0.0031
Vein	0.0045	±0.0011	0.0023	±0.0022

* The A-V difference is not significant, $p > 0.05$.
† The A-V difference is significant, $p < 0.001$. The values of infused NE are derived by subtracting the fluorescence of the appropriate control samples from the total fluorescence of the samples obtained during infusion.

TABLE VI

Hepatic A-V differences of infused NE and the plasma material measured as NE

	Plasma material measured as NE in 14 subjects		Infused NE in 5 subjects*	
	Mean	SD	Mean	Range
Artery	0.0036†	±0.0020	0.0220	0.0132–0.0280
Vein	0.0020	±0.0013	0.0013	0.0002–0.0035

* Infusions ranged from 7 to 26 μg NE per minute.
† The A-V difference is significant, $p < 0.01$.

Arteriovenous differences across the liver were also compared. The subjects were 14 patients who underwent diagnostic right heart catheterization for valvular heart disease. None had clinically evident liver disease. Liver function tests were either normal or mildly abnormal in a pattern consistent with mild congestive heart failure. Data are summarized in Table VI. Values for the NE-like material were only 55 per cent as high in hepatic venous plasma as in simultaneously sampled arterial plasma. Appreciable concentrations (average, 0.0020 μg per 5 ml) of the NE-like material remained in the hepatic venous blood of most patients. Five patients were given infusions of NE into an antecubital vein at rates varying from 7.0 to 26.0 μg per minute, the average rate having been 14.2 μg per minute. Pronounced elevations in arterial NE occurred in every patient but elevations in hepatic venous blood were much lower, averaging only 6 per cent of the arterial level. The average level of infused NE in the hepatic venous blood was less than the level of the NE-like material present before the infusion.

The endogenous material and infused NE were different in the following respects: *1*) 94 per cent of the infused NE but only 45 per cent of the endogenous NE-like material was removed in the liver; *2*) 72 per cent of the infused NE and none

of the endogenous material was removed in the leg.

DISCUSSION

Material forming fluorescent products with EDA having the pattern of those formed by NE is present in normal arterial plasma in an average concentration of 0.72 μg per L (0.0036 μg per 5 ml). When NE is infused intravenously at the rate of 4 μg per minute, the average concentration of material in arterial blood having the fluorescent pattern of NE is increased by 200 per cent; the average concentration in venous blood is increased by 50 per cent. The NE-like material in normal arterial plasma has an A-V difference across the extremity of − 0.08 μg per L (− 0.0004 μg per 5 ml). This difference requires over 47 determinations to reach a significant p value. The NE added by the infusion always has a positive A-V difference, and in some instances none of it appears in femoral venous blood. The splanchnic A-V difference for infused NE is greater than that for the material in normal plasma.

The material in normal plasma having the fluorescent characteristics of NE is present in equal amounts in blood from the femoral and antecubital veins. At low levels of infusion the concentration of NE in femoral venous blood is less than in antecubital venous blood. The concentration of the material in normal plasma is the same in blood draining the forearm and hand as in blood draining only the forearm.

These observations raise the question of whether there is a measurable quantity of NE in arterial and venous blood. At a concentration of 0.72 μg per L most of the arterial NE would be removed in the leg and arm. If the material thought to be NE is indeed NE, it appears reasonable to assume that NE is removed from the arteriolar and capillary bed in one area and added back to the blood by diffusion from the nerve endings of the autonomic nervous system at a site distal to the area of removal. In normal subjects the amount removed and the amount added would appear to be in remarkably close balance, since an A-V difference of NE-like material is not demonstrable in many subjects, although a small difference can be shown in a large series. Furthermore, the rates of removal and addition would seem to be such that the venous blood from such diverse tissues as the forearm and hand contained the same concentration of NE.

It has generally been assumed that the NE found in the urine after removal of the adrenal medullae comes from NE made at adrenergic nerve endings. A further assumption has been that the NE reached the kidneys by way of the blood. There is no good evidence to support this second assumption, as the NE in question could be made in the kidneys themselves. NE in the urine may reflect adrenergic activity not of the whole adrenergic system but of the sympathetic nerve endings in the kidney. In view of the observations recorded in this paper, further investigation of the source of urinary NE is needed.

SUMMARY AND CONCLUSIONS

The ethylenediamine method has been modified to improve its sensitivity and to make it more quantitative. The concentration in normal arterial plasma of material reacting chemically like norepinephrine (NE) is 0.72 μg per L (0.0036 μg per 5 ml), and reacting chemically like epinephrine (E) is 0.10 μg per L (0.0005 μg per 5 ml). The material called NE has an A-V difference across the extremity of − 0.08 μg per L (− 0.0004 μg per 5 ml). It has the same concentration in blood collected from the forearm and hand as in blood from the forearm alone. Its concentration in hepatic venous blood is 55 per cent of that present in arterial blood.

NE infused at a rate of 4 μg per minute has an average arterial concentration of 1.62 μg per L (0.0081 μg per 5 ml) and an average concentration in femoral venous blood of 0.46 μg per L (0.0023 μg per 5 ml). It is frequently absent in femoral venous blood. Hepatic venous blood contains only 6 per cent of that present in arterial blood.

Either the material measured in normal plasma is not NE, or NE is removed in one part of the circulation of the forearm and leg and added back to the venous blood in approximately the same concentration at a more distal point in the circulation.

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THE ROLE OF INTERMEDIARY CARBOHYDRATE METABOLISM IN REGULATING ORGANIC IODINATIONS IN THE THYROID GLAND *

By GEORGE C. SCHUSSLER AND SIDNEY H. INGBAR† WITH THE TECHNICAL ASSISTANCE OF PATRICIA EVELETH

(From the Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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As pointed out in recent reviews (1, 2), the synthesis of hormonally active materials by the thyroid gland proceeds via a sequence of seemingly discrete reactions. These may be categorized as follows: concentration of inorganic iodide from the extracellular fluid, oxidation and organic binding of iodine to yield iodotyrosines, and coupling of iodotyrosines to form the hormonally active iodothyronines. The latter two groups of reactions occur within the matrix of the thyroglobulin molecule, a large molecular protein for whose synthesis and storage the thyroid gland is especially adapted.

It seems clear that ultimately the energy for these synthetic processes, as well as for the maintenance of glandular architecture, must derive from metabolic sources. However, little is known of how the energy released during intermediary metabolism is imparted to those reactions involved in hormonal biosynthesis. Previous studies have indicated that the iodide-concentrating function of the thyroid is dependent on oxidative processes leading to the formation of high energy phosphate bonds (3, 4). The present studies were undertaken to determine whether a comparable link to metabolic events could be delineated in the case of the next stage in hormonal synthesis, the oxidation of iodide and formation of monoiodotyrosine. From the results obtained, a metabolic sequence is postulated whereby the oxidation of iodide is regulated by glucose metabolism via reactions coupled to pyridine nucleotide-linked dehydrogenations. Evidence is presented which suggests that the effects of thyrotropin (TSH) on organic iodinations in the thyroid may be mediated by an enhancement of glucose metabolism within the gland.

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† Investigator, Howard Hughes Medical Institute.

METHODS

Experiments were performed with slices or homogenates prepared from thyroid glands which had been obtained at the abattoir from freshly killed sheep or lambs and brought to the laboratory packed in ice. Throughout the preparative procedure, the tissues were chilled at 0 to 4° C.

Preparation of homogenates. Several thyroid glands were employed in the preparation of a single homogenate. Thyroids were stripped of fat and fascia, minced with a Stadie-Riggs blade and homogenized with a Potter-Elvehjem glass homogenizer in 5 vol of a medium containing phosphate buffer (pH 7.4), 6×10^{-3} M; $MgSO_4$, 3×10^{-3} M; KCl, 0.14 M. Usually, homogenates were rehomogenized with a tight-fitting Teflon homogenizer prior to incubation. No further efforts were made to eliminate intact cells. Since it was the purpose of these experiments to relate organic iodination to carbohydrate metabolism, the medium chosen was identical in terms of inorganic constituents to that described by Wenner, Dunn and Weinhouse as supporting glycolysis in cell-free systems (5). Aliquots of homogenate (1.7 ml) were pipetted into the main compartment of Warburg vessels. In many experiments, the sodium salt of glucose-6-phosphate [1] was added to a final concentration of 0.015 M. After gassing of flasks had been completed and

[1] The following abbreviations will be used in text, tables and illustrations: glucose-6-phosphate, G-6-P; triphosphopyridine nucleotide (oxidized), TPN; triphosphopyridine nucleotide (reduced), TPNH; diphosphopyridine nucleotide (oxidized), DPN; diphosphopyridine nucleotide (reduced), DPNH; flavin adenine dinucleotide (oxidized), FAD; flavin mononucleotide (oxidized), FMN; glutathione (oxidized), GSSG; glutathione (reduced), GSH; methylene blue (oxidized), MB; norethylmaleimide, NEM.

temperature equilibration achieved, the final volume of homogenate was brought to 2 ml by tipping into the main compartment 300 μl of medium containing 23 μc of carrier-free inorganic I^{131},[2] and the substrates, cofactors, enzymes or inhibitors to be tested. CO_2 was absorbed by 300 μl of 10 per cent KOH in another side-arm or in the center well. Incubation was usually carried out for 1 hour in a Warburg apparatus at 38° C, under an atmosphere of 100 per cent oxygen. A few experiments were performed in Erlenmeyer flasks under oxygen in a metabolic shaker.

In certain experiments, an alternative method was employed. When materials to be tested had limited solubility, these were dissolved together with the radioactive iodide in 1 ml of medium. A more concentrated homogenate was then added to achieve a final homogenate of the usual volume and concentration. A similar procedure was followed in homogenate experiments with C^{14}-labeled glucose [3] in order that the exact quantity of radioactivity added to each vessel could be determined. In the latter experiments, the final concentration of glucose was 0.3 to 0.5 mg, containing 1 μc of glucose-C^{14}, per ml.

Preparation of slices. Thyroid slices, rather than homogenates, were employed in experiments designed to assess the effects of TSH on the metabolism of C^{14}-labeled glucose. Slices, 50 to 100 mg in weight, were cut with a Stadie-Riggs microtome and were incubated in 2 ml of Krebs-Ringer-phosphate medium (KRP) containing 1 μc of C^{14}-labeled and 1.5 mg of stable glucose per ml. TSH, 1 U,[4] was added to each experimental vessel in 100 μl of KRP, and a similar volume of KRP was added to the controls. Incubation was carried out at 38° C under 100 per cent oxygen in a Warburg apparatus. CO_2 was absorbed by 300 μl of 10 per cent KOH in a side-arm. Similar experimental conditions were employed to assess the effects of oxidized methylene blue (MB) on glucose metabolism.

Measurement of I^{131}-organification. After incubation, the reaction was stopped by chilling each vessel and adding 25 μl of 0.08 M methimazole. The percentile organification of iodide was usually determined by precipitation with trichloroacetic acid (TCA). Aliquots of homogenate (20 μl) were added to 0.7 ml of a solution of human serum albumin (1.4 g per 100 ml) in 13 × 100 mm test tubes. Two ml of 10 per cent TCA was then added, the resulting precipitate was washed three times with 5 per cent TCA, dissolved in 2 N NaOH, and counted directly in a well-type scintillation counter. Pooled supernatants were alkalinized to prevent loss of iodide, diluted to standard volume and counted. Percentile organification was calculated according to the following formula:

$$I^{131}\text{ organified (per cent)} = \frac{(I^{131}\text{ in precipitate}) \times 100}{(I^{131}\text{ in precipitate}) + (I^{131}\text{ in pooled supernatant})}.$$

Chromatographic methods were used to characterize the nature of the iodinated products formed by the homogenate. Aliquots of homogenate (20 μl) together with carrier iodide, mono- and diiodotyrosine (MIT and DIT), were applied directly to strips of Whatman no. 1 filter paper. Following chromatography in butanol : dioxane : 2 N ammonia (4 : 1 : 5) and in butanol : 2 N acetic acid (1 : 1) systems,[5] radioautographs were prepared. Carrier compounds were then localized by spraying with diazotized sulfanilic acid for MIT and DIT, and with palladium chloride for iodide. For quantitative analysis, radioactive zones located by radioautography were cut from unstained chromatographic strips and were counted in a well-type scintillation counter.

In both chromatographic systems, three major zones of radioactivity were consistently seen in chromatograms of unhydrolyzed homogenate: a band at the point of application (origin material), a band coinciding with carrier inorganic iodide, and a less well defined band seen at the solvent front (front material). The latter comprised a variable proportion (30 to 60 per cent) of the non-inorganic I^{131}. A band between iodide and front material, similar to the Unknown 2 (U_2), first described by Taurog, Tong and Chaikoff (8), was occasionally seen but never constituted a significant portion of the total I^{131}. After tryptic hydrolysis of the homogenate, the radioactivity at the origin decreased and radioactivity appeared in the zone of carrier MIT. This indicated that the radioactive material at the origin was primarily protein containing MIT in peptide linkage. Unlike the origin material, iodinated material at the solvent front was readily eluted from chromatographic strips by chloroform, methanol, ether and butanol. Following rechromatography of the eluted front-running material, the radioactivity again appeared at the solvent front and coincided with an area which stained with Sudan black. These findings indicated that the front-running material was an iodinated lipid, suggesting that,

[2] Inorganic I^{131} was obtained from Abbott Laboratories, Oak Ridge, Tenn., and was free, by paper chromatography, of noniodide contaminants described by other workers (6).

[3] C^{14}-labeled glucose was obtained from the New England Nuclear Corp., Boston, Mass. Samples were apparently pure according to radioactive scans of chromatographic strips. Radioautography of samples chromatographed in butanol : acetic acid : water (78 : 10 : 12) occasionally revealed a faint band in the zone occupied by labeled lactic acid. Direct counting of these zones revealed that this area contained no more than 0.1 per cent of the total counts on the strip (7). Measurement of this contaminant as $C^{14}O_2$ could not have accounted for a significant proportion of the acid volatile counts produced by homogenates under the influence of stimulatory cofactors.

[4] Thytropar, Armour.

[5] Chromatographic solvents were enriched with propylthiouricil in order to prevent oxidation of compounds during chromatography.

in the homogenate system, oxidation of iodide results in the iodination of both proteins and lipid.

Chromatography was also performed to validate the use of TCA-precipitation as a measure of organified iodine. Direct chromatography of the TCA-precipitate showed radioactivity at both the origin and solvent front, but not in the iodide area. Chromatography of TCA-supernatants revealed only inorganic iodide. A chloroform : methanol extract of the TCA-precipitate contained only front-running material; the residual I^{131} in the precipitate was entirely composed of origin material. A saline extract of the precipitate showed only origin material. The ability to extract the front material prior to chromatography supported the conclusion that the front material was lipid iodinated in the homogenate, rather than a chromatographic artifact. Thus, both front and origin material appeared to be true products of iodination in the homogenate system. Since both were found in TCA-precipitates and not in supernatants, the precipitation technique could be considered as valid as chromatography for the assessment of the organification of radioiodide.

$C^{14}O_2$ production. The $C^{14}O_2$ evolved during incubation of tissues with labeled glucose was absorbed in 300 μl of KOH in the side-arm of Warburg flasks. At the end of the incubation, tissues and media were removed and replaced by 2 ml of 5 M H_2SO_4. Two hundred μl of 0.5 M Hyamine was placed in the center well and the flasks were tightly stoppered. The acid and alkali were then thoroughly mixed and the flasks were gently shaken at room temperature for 30 minutes. The Hyamine, now containing CO_2 released from the alkali, together with several washes of the center well, was transferred to glass counting vials containing liquid scintillator. Samples were cooled overnight and then counted in an automatic liquid scintillation counter. An aliquot of the original incubation medium was counted as a standard. Internal standards were used to correct for variations in counting efficiency. The quantity of $C^{14}O_2$ produced was expressed as a per cent of added C^{14}-labeled glucose per gram of tissue.

Miscellaneous methods. The reduction of added pyridine nucleotide coenzymes was assessed by measuring the optical density at 340 mμ of supernatants obtained from 1 : 3 (vol/vol) mixtures of homogenate and 95 per cent ethanol (9). When necessary, Celite was added as a clearing agent. Analyses for stable glucose were performed by an enzymatic method (10).

RESULTS

Duplicate or triplicate vessels were incubated in all experiments. Results presented represent the mean of individual determinations, which, with few exceptions, agreed closely.

Organic iodinations

Anoxia (Table I). Despite wide variations in percentile iodinations obtained in control vessels, organic iodinations were markedly depressed by anaerobic incubation. Comparable inhibition of iodination was effected by anoxia, even in the presence of factors which were highly stimulatory to iodinations when oxygen was present. At lower partial pressures, the system was especially sensitive to the partial pressure of oxygen, but maximal rates of iodination were not achieved until the atmosphere was 100 per cent oxygen (Figure 1).

TABLE I

The effect of anoxia on organic iodinations in plain and supplemented homogenates of sheep thyroid glands

Expt. no.	Compound added*	Organic I^{131}	
		O_2	N_2
		% total	
4	0	8.4	0.8
15	0	1.6	0.5
18	0	3.9	0.5
24	0	3.1	0.6
	DPN	2.8	0.5
33	0	2.4	0.5
	DPN	2.3	0.4
	TPN	3.7	0.4
	TPNH	3.2	0.4
110	0	0.9	0.3
	FAD	1.3	0.4
	TPN + FAD	6.2	0.6
34	0	11.1	0.4
	$CuCl_2$	39.8	0.9
	$HgCl_2$	33.0	1.8
42	MB	5.5	0.3

* Compounds added to a final concentration of 0.0007 M, except in the following instances: $CuCl_2$, 0.0125 M; $HgCl_2$, 0.0005 M; MB, 0.00018 M.

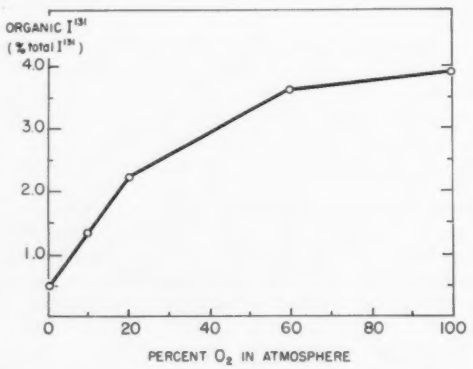

FIG. 1. THE EFFECT OF VARYING OXYGEN TENSIONS ON ORGANIC IODINATIONS IN A HOMOGENATE OF SHEEP THYROID GLANDS.

TABLE II

The effects of dialysis and storage on organic iodinations in homogenates of sheep thyroid glands *

	Organic I^{131}		
	Control	Dialyzed	Stored
	% total	*% total*	*% total*
1	1.3	8.2	2.9
3	4.9	11.3	7.8
4	8.4	29.2	9.4
9	5.4	30.0	
9a†	3.3	25.0	
16	1.3	13.9	
18	3.9		4.5
22	1.1	2.1	
22a†	0.9	1.7	
26	2.8	13.5	
60	2.7		3.7
63	3.3	22.2	
68	1.5	20.9	
69	1.9	24.4	

* An aliquot of the control homogenate either stored or dialyzed for 16 hours against 25 vol of homogenization medium at 4° C prior to incubation with inorganic I^{131}.

† Glucose-6-phosphate (0.015 M) added to control and dialyzed homogenates prior to incubation.

Dialysis and storage (*Table II*). Dialysis of homogenate for 16 hours at 4° C against 24 vol of the homogenization medium consistently increased the percentage of added I^{131} organified. In 12 experiments, percentile iodinations in dialyzed homogenates averaged 650 per cent of the values obtained the day before in undialyzed controls. Iodinations in homogenates stored at 4° C for the same period of time were also consistently, although less markedly, stimulated.

Sulfhydryl inhibitors. The addition of $CuCl_2$ markedly stimulated organic iodinations. In eight experiments, iodinations in the presence of $CuCl_2$ (0.0125 M) averaged 692 per cent of control values. In four experiments, performed in the same homogenates before and after dialysis, the stimulatory effect of $CuCl_2$ was abolished or greatly diminished in the dialyzed samples (Table III).

The addition of $HgCl_2$ also produced a pronounced stimulation of iodinations which was abolished by prior dialysis of the homogenate (Table III).

NEM, a specific sulfhydryl inhibitor (11), was stimulatory at concentrations between 2×10^{-4} and 10^{-3} M; higher concentrations were inhibitory (Figure 2). After dialysis of the homogenate, stimulatory effects of NEM were diminished or changed to inhibition (Figure 3).

Glutathione. Iodinations in the homogenate system were inhibited by the addition of glutathione. When compared in the same homogenates, 0.02 M GSH was more inhibitory than an equivalent concentration of GSSG. In other experi-

TABLE III

The effects of heavy metals on organic iodinations in homogenates of sheep thyroid glands

Expt. no.	Concentration	Organic I^{131}	
		Control	Inhibitor
	M	*% total*	
$CuCl_2$			
34	0.0125	11.1	39.8
66	0.0125	9.0	20.2
76	0.0125	2.3	24.0
79	0.0125	2.8	26.6
109	0.0125	3.6	14.7
4	0.0025	8.4	8.3
	0.0040	8.4	14.0
	0.040	8.4	7.2
4-D*	0.0040	29.2	17.0
26	0.0005	2.8	3.2
	0.0025	2.8	8.3
	0.0125	2.8	16.6
26-D	0.0125	13.5	11.3
68	0.0125	1.5	13.9
68-D	0.0125	20.9	31.6
69	0.0125	1.9	18.3
69-D	0.0125	24.4	31.4
$HgCl_2$			
34	0.0005	11.1	33.0
26	0.0005	2.8	27.1
	0.0025	2.8	4.7
	0.0125	2.8	3.2
26-D	0.0005	13.5	12.6

* D indicates homogenate dialyzed against 25 vol of homogenization medium for 16 hours at 4° C prior to addition of inhibitor and incubation with I^{131}. Effects of inhibitor had also been tested in the same homogenate prior to dialysis.

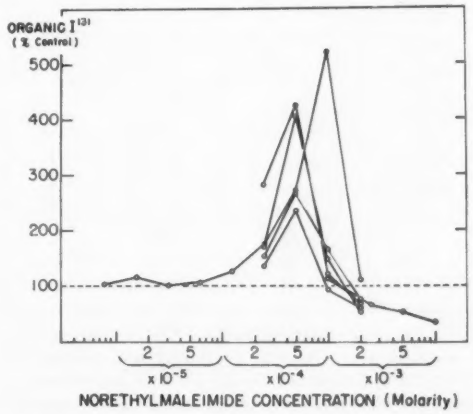

FIG. 2. THE EFFECT OF VARYING CONCENTRATIONS OF A SULFHYDRYL INHIBITOR, NORETHYLMALEIMIDE, ON ORGANIC IODINATIONS IN HOMOGENATES OF SHEEP THYROID GLANDS.

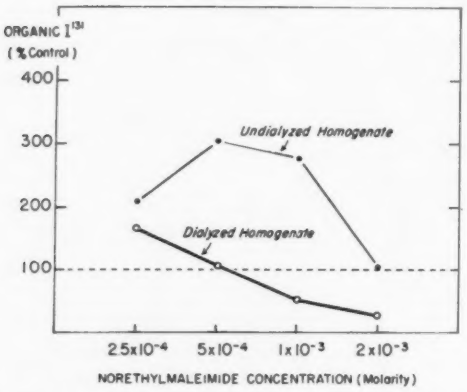

FIG. 3. THE EFFECT OF DIALYSIS OF SHEEP THYROID HOMOGENATES ON THE RESPONSE OF ORGANIC IODINATIONS TO THE ADDITION OF NORETHYLMALEIMIDE. Iodinations were measured prior to and following dialysis of homogenate at 4° C against 24 vol of homogenization medium for 16 hours. In each instance, the control value is that obtained in appropriately treated homogenate not supplemented with norethylmaleimide.

ments, the inhibitory effect of GSSG was inconstant. However, when TPN (7×10^{-4} M) was added, iodinations in the presence of GSSG were more often lower than those found in control, unsupplemented vessels (Figure 4). The consistent and pronounced inhibitory effect of GSSG

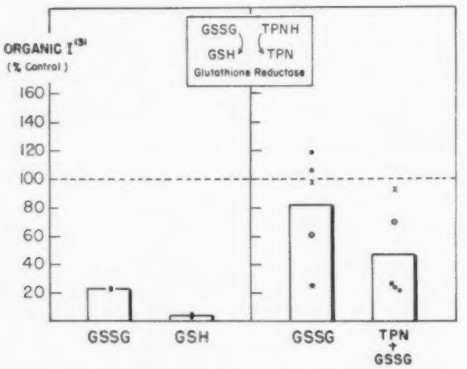

FIG. 4. EFFECT OF OXIDIZED AND REDUCED GLUTATHIONE ON ORGANIC IODINATIONS IN SHEEP THYROID HOMOGENATES AND THE EFFECT OF ADDED TPN ON THE RESPONSE TO OXIDIZED GLUTATHIONE. Oxidized and reduced glutathione are compared directly in two experiments, as shown on the left. Enhancement of the response to oxidized glutathione by TPN is shown on the right. Each symbol represents results obtained in an individual experiment. Schema in enclosed box depicts enzymatic pathway for the reduction of oxidized glutathione.

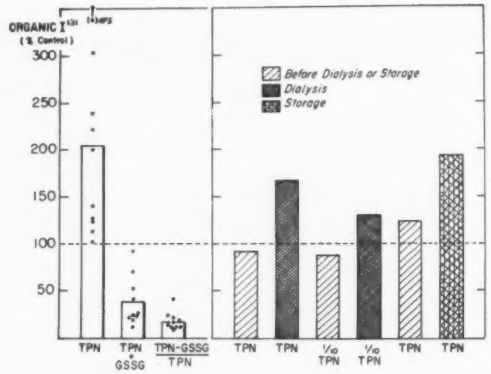

FIG. 5. MODIFICATION OF THE TPN EFFECT ON ORGANIC IODINATIONS BY GLUTATHIONE AND BY A LABILE ENDOGENOUS INHIBITOR. Shown on the left are results of ten experiments in which the effects of TPN, with and without added oxidized glutathione, were compared. TPN–GSSG/TPN represents ratio of iodinations obtained in the same homogenate under the two conditions, expressed as a per cent. On the right are shown effects of dialyzing or storing homogenates on the response to TPN. Dialysis experiment performed in a single homogenate; storage experiment in another. Values expressed as a per cent of control value obtained in either fresh, dialyzed or stored homogenates, not supplemented with TPN.

in the presence of TPN was best demonstrated, however, when iodinations in vessels containing both cofactors were compared with those obtained with TPN alone (Figure 5).

Glycolytic substrates (*Table IV*). A number of substrates was tested. The addition of succinate consistently stimulated iodination. Other substrates did not seem to have a marked or consistent effect.

Pyridine nucleotide coenzymes. Both TPN (7×10^{-4} M) and DPN (7×10^{-4} M) usually stimulated iodinations when added to thyroid homogenates (Figure 6). In 45 experiments, organic iodinations in TPN-enriched homogenates averaged 182 per cent of control values. The effect of DPN was less marked and less consistent. In 24 experiments DPN increased iodinations by an average of only 27 per cent, and in 7 of these experiments there was no stimulatory effect. The impression that the effect of TPN was more pronounced than that of DPN was confirmed by comparing effects of the coenzymes in the same homogenate. In each of 20 such experiments, TPN was the more stimulatory and mean iodination in TPN-supplemented flasks was 153 per cent of

TABLE IV

The effects of glycolytic substrates on organic iodinations in homogenates of sheep thyroid glands

Expt. no.	Substrate*	Organic I^{131} Control	Organic I^{131} Substrate
		% total	
31	Glucose	3.0	2.7
32	Glucose	1.7	1.9
4	G-6-P	8.4	5.1
9	G-6-P	5.4	3.3
9-D	G-6-P	30.0	25.0
22	G-6-P	0.9	1.1
38	G-6-P	1.8	2.0
39	G-6-P	2.5	2.3
35	Fructose	1.1	0.8
58	Pyruvate	17.6	18.2
37-D	Citrate	58.6	60.1
58	Citrate	17.6	15.2
40	Isocitrate	0.6	1.0
40	α-Ketoglutarate	0.6	0.6
38	Succinate	1.8	3.1
39	Succinate	2.5	3.7
40	Oxaloacetate	0.6	0.8
58	Oxaloacetate	17.6	21.9

* All substrate added to a final concentration of 0.02 M, except glucose-6-phosphate, which was added to a final concentration of 0.015 M.

that obtained in vessels supplemented with DPN. The rate of reduction of added TPN in a homogenate containing G-6-P is compared to the rate of organic iodinations in Figure 7. Following the addition of TPN, the concentration of TPNH rapidly increased, reaching a virtually constant value within 5 minutes. Iodinations, however, continued at an accelerated rate for the remainder of the experiment. Although a second addition of TPN again resulted in a rapid increase in the

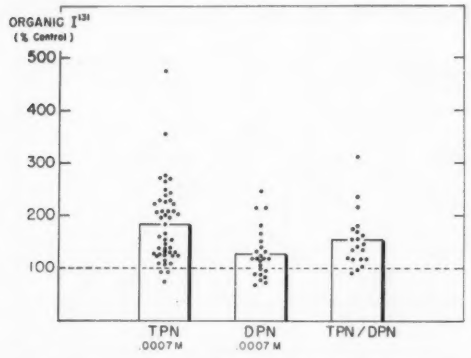

FIG. 6. EFFECTS OF TPN AND DPN ON ORGANIC IODINATIONS IN SHEEP THYROID HOMOGENATES. TPN/DPN represents ratio, expressed as per cent, of values found in 20 experiments in which effects of TPN and DPN were compared in the same homogenate.

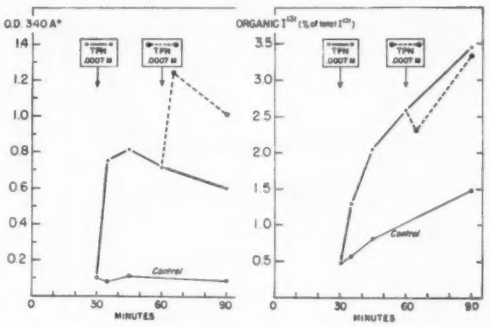

FIG. 7. THE RELATION BETWEEN ORGANIC IODINATIONS AND THE REDUCTION OF TPN IN A SHEEP THYROID HOMOGENATE. At the indicated times, TPN tipped into homogenates from the side-arm of Warburg vessels in quantities sufficient to enhance the concentration of TPN by 0.0007 M.

concentration of TPNH, there was no further stimulation of iodinations. Following the addition of comparable concentrations of DPN, the increase in concentration of DPNH was far less than had been the case with TPN.

The effects of TPN and DPN were modified by supplementing homogenates with reducing systems specifically requiring one of the coenzymes

TABLE V

Modification of the stimulatory effects of pyridine nucleotide coenzymes on organic iodinations by the addition of their specific reducing systems

Compound added	Concentration*	Percentile organic iodinations (% control iodinations)
DPN	0.0007	118
DPN Ethanol	0.0007 0.01	95
DPN Ethanol Alcohol dehydrogenase†	0.0007 0 01 0.023	87
TPN Isocitrate Isocitric dehydrogenase‡	0.0007 0.02 1.0	127 121 100
TPN Isocitrate Isocitrate dehydrogenase	0.0007 0.02 1.0	152

* Molar concentrations of all compounds except alcohol and isocitric dehydrogenases, for which concentrations given represent mg/ml homogenate.

† Cryst. from yeast, Sigma Chemical Co., 100 to 150,000 U/mg.

‡ Type I, crude, Sigma Chemical Co., 30 U/mg.

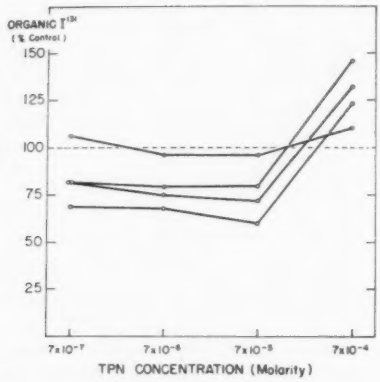

FIG. 8. THE EFFECTS OF VARYING CONCENTRATIONS OF TPN ON ORGANIC IODINATIONS IN SHEEP THYROID HOMOGENATES.

as hydrogen acceptor. The addition of isocitric acid and isocitric dehydrogenase potentiated the stimulatory effect of TPN on iodinations; the addition of ethyl alcohol plus alcohol dehydrogenase changed the stimulatory effect of DPN to a moderate inhibition. Similar results were obtained in several experiments, one of which is shown in Table V.

When added to the same homogenates in which the usual concentration of TPN (7×10^{-4} M) was stimulatory, lower concentrations of TPN (7×10^{-5} to 7×10^{-7} M) failed to stimulate iodinations and, indeed, were moderately inhibitory (Figure 8). The effects of DPN (7×10^{-4} M), and of TPN at 0.1 its usual concentration (i.e., 7×10^{-5} M), alone and in combination, are compared in Figure 9. The addition of both coenzymes produced a stimulation greater than that expected from the sum of their individual effects, and iodinations were then usually equal to or greater than those obtained with the higher concentrations of TPN alone.

Prior dialysis of homogenates altered the response to TPN. Representative results are shown in Figure 5. In a homogenate in which the full concentration of TPN (7×10^{-4} M) was not stimulatory prior to dialysis, stimulation was produced by TPN after the homogenate had been dialyzed. The usual inhibitory effect of lower concentrations of TPN (7×10^{-5} M) was also changed to stimulation by dialysis. In separate experiments, a similar potentiation of the response to TPN was achieved by storage.

When TPN (7×10^{-4} M) was added to homogenates containing stimulatory concentrations of NEM, the resulting increase in iodinations was considerably greater than the stimulation produced by the addition of TPN alone (Figure 10). Even concentrations of TPN which were inhibitory when added alone (7×10^{-5} M) became stimulatory when added together with NEM. The addition of GSSG abolished the stimulatory effect of TPN and produced a consistent inhibition of iodinations, as compared with iodinations obtained in vessels supplemented with TPN alone (Figure 5).

Methylene blue (*Table VI*). In seven separate homogenates to which MB (1.8×10^{-4} M) was added, iodinations were increased by an average of 94 per cent. The addition of TPN

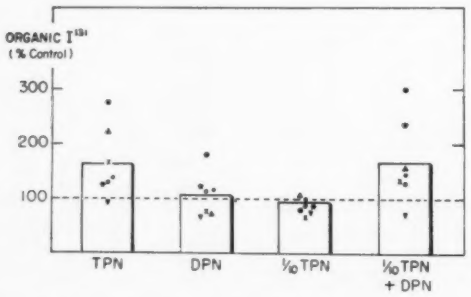

FIG. 9. SYNERGISTIC EFFECTS OF TPN AND DPN ON ORGANIC IODINATIONS IN SHEEP THYROID HOMOGENATES. TPN and DPN, final concentration 0.0007 M.; 0.1 TPN, final concentration 0.00007 M. Each symbol represents results obtained in a single experiment.

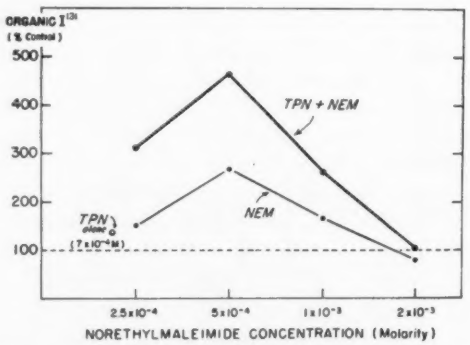

FIG. 10. THE EFFECT OF TPN AND NORETHYLMALEIMIDE, ALONE AND IN COMBINATION, ON ORGANIC IODINATIONS IN A SHEEP THYROID HOMOGENATE.

TABLE VI

The effects of methylene blue and cytochrome C on organic iodinations in homogenates of sheep thyroid glands

Expt. no.	Organic I^{131} Control	Organic I^{131} Compound added
	% *total*	
Methylene blue (0.00018 M)		
42	2.5	5.5
42a*	5.2	10.1
45	1.3	3.5
66	9.0	11.9
68	1.5	2.2
68-D†	20.9	38.6
69	1.9	4.8
69-D	24.4	32.2
76	2.3	4.4
78	3.5	5.1
Cytochrome C (0.0007 M)		
57	2.4	1.6
68	1.5	2.1
68-D	20.9	26.4
69	1.9	1.8
69-D	24.4	24.7
76	2.3	3.7

* Homogenate supplemented with TPN (0.0007 M).
† D indicates homogenates dialyzed against 25 vol of homogenization medium for 16 hours at 4° C prior to addition of compound and incubation with I^{131}. Effects of compound also tested in same homogenate prior to dialysis.

with MB produced a further stimulation of iodinations.

Flavin coenzymes. FAD had a weak and inconstant stimulatory effect, producing an average increase of 22 per cent in organic iodinations in seven experiments. In the same homogenates,

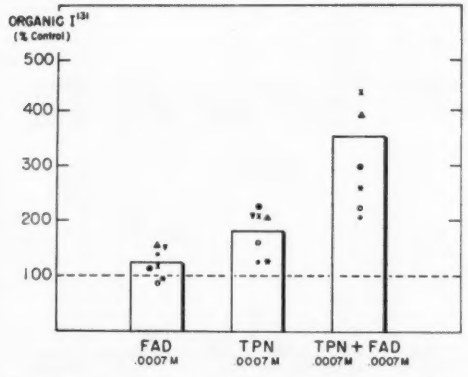

FIG. 11. SYNERGISTIC EFFECTS OF TPN AND FAD ON ORGANIC IODINATIONS IN SHEEP THYROID HOMOGENATES. Concentrations shown represent final concentration of added compound in homogenate. Each symbol represents the results of an individual experiment.

TABLE VII

The effect of light on organification of I^{131} in a solution of human serum albumin and in a homogenate of sheep thyroid glands

Expt. no.	Cofactor added*	Conditions of experiment	Organic I^{131} No cofactor	Organic I^{131} Cofactor
			% *total*	
Incubation in human serum albumin (2.5 g/100 ml)				
100	FAD	Dark	2.0	2.1
	FMN	Dark	2.0	2.6
	FAD	Light	2.7	4.1
	FMN	Light	2.7	6.9
108	TPN	Light	0.8	0.7
	FAD	Light	0.8	2.8
	TPN+FAD	Light	0.8	2.2
	TPN	Light-N_2†	0.6	0.6
	FAD	Light-N_2	0.6	1.0
	TPN+FAD	Light-N_2	0.6	0.8
Incubation in thyroid homogenate				
110	TPN	Dark	0.9	1.9
	TPN	Light	0.7	1.9
	FAD	Dark	0.9	1.3
	FAD	Light	0.7	2.6
	TPN+FAD	Dark	0.9	5.8
	TPN+FAD	Light	0.7	6.2
	FAD	Light-N_2	0.3	0.4
	TPN+FAD	Light-N_2	0.3	0.6

* Cofactors added to a final concentration of 0.0007 M.
† N_2 indicates incubations performed in atmosphere of 100% nitrogen. All others performed in 100% oxygen.

TPN alone increased iodinations by an average of 80 per cent. However, when the two coenzymes were added together, iodinations were increased by 252 per cent (Figure 11). In a single experiment, FMN stimulated iodinations moderately.

Cytochrome C (Table VI). Cytochrome C had only a slight or inconstant effect on organic iodinations, whether added alone or with other cofactors.

Effects of light (Table VII). Iodinations in the tissue-free system were markedly increased by the addition of FAD or FMN when flasks were incubated in room light reinforced by an ultraviolet lamp. This effect was greatly diminished by the exclusion of light or oxygen. Even in the presence of light and oxygen, TPN did not stimulate iodinations in the tissue-free system, nor did it enhance the stimulatory effect of FAD.

In thyroid homogenates, the presence or absence of light had no effect upon control iodinations. The increase in iodinations which followed the addition of FAD was partially inhibited in the dark. On the other hand, the increase in organic iodinations due to TPN, as well as the marked synergism between TPN and FAD, was unaffected by the presence or absence of light.

Metabolic inhibitors (Table VIII). KCN inhibited organic iodinations. Antimycin A had a very slight stimulatory effect when added alone

TABLE VIII

The effect of metabolic inhibitors and antithyroid agents on organic iodinations in homogenates of sheep thyroid glands

Expt. no.	Compound added	Concentration	Organic I^{131} Control	Organic I^{131} Inhibitor
		M	*% total*	
Metabolic inhibitors				
4	KCN	0.010	8.4	0.8
49	KCN	0.010	3.8	0.1
52	KCN	0.010	4.8	2.1
30	2,4-Dinitrophenol	0.00010	6.5	6.7
		0.00025	6.5	6.5
		0.00050	6.5	6.6
		0.0010	6.5	6.5
38	Malonate	0.010	1.8	3.2
39	Malonate	0.010	2.5	3.3
71	Iodoacetate	0.010	1.4	0.2
	Norethylmaleimide	0.010	1.4	0.5
80	Anitmycin A	0.000003	2.4	2.7
81	Antimycin A	0.000003	1.4	1.5
Antithyroid agents				
36	Propylthiouracil	0.010	2.2	0.3
	Perchlorate	0.001	2.2	2.3
		0.010	2.2	2.2
	Thiocyanate	0.050	2.2	0.2
49	Methimazole	0.003	3.8	0.2

or together with TPN or DPN. Dinitrophenol had no effect. In two experiments, malonate (0.02 M) stimulated iodinations.

Antithyroid drugs (Table VIII). Methimazole, propylthiouracil and thiocyanate greatly inhibited iodinations; perchlorate had no effect.

Glucose metabolism

The effect on glucose metabolism of various factors which stimulate organic iodinations is shown in Table IX. As compared with slices, unsupplemented homogenates metabolized glucose to CO_2 at a relatively slow rate. During 1 hour of incubation, the $C^{14}O_2$ recovered from glucose labeled in the 1 and 6 positions (1-$C^{14}O_2$ and 6-$C^{14}O_2$) averaged 0.23 and 0.04 per cent per g of tissue, respectively, in eight experiments. In seven experiments with slices incubated for a comparable period, the production of 1-$C^{14}O_2$ averaged 3.86 per cent and 6-$C^{14}O_2$ 1.53 per cent of added C^{14}-labeled glucose per g of tissue.

TABLE IX

The effect of factors stimulatory to organic iodinations on the production of carbon dioxide from glucose of homogenates of sheep thyroid glands

Expt. no.	Compound added	$C^{14}O_2$ production (% added glucose/g tissue) Control 1-$C^{14}O_2$	Control 6-$C^{14}O_2$	Compound added 1-$C^{14}O_2$	Compound added 6-$C^{14}O_2$
96	DPN	0.09	0.01	2.33	0.18
	DPN+TPN*	0.09	0.01	6.94	
97	DPN	0.14	0.02	1.57	0.04
	TPN	0.14	0.02	1.41	0.12
	TPN*	0.14	0.02	0.31	0.02
	DPN+TPN*	0.14	0.02	8.70	0.08
101	DPN	0.23	0.01	1.64	0.02
102	TPN	0.44	0.10	0.82	0.16
	FAD	0.44	0.10	0.58	0.15
	TPN+FAD	0.44	0.10	9.10	0.25
103	TPN	0.08	0.01	0.47	0.05
	TPN+FAD	0.08	0.01	10.08	0.12
	TPN+FMN	0.08	0.01	10.77	0.17
105	TPN	0.49	0.07	3.86	0.04
	TPN+FAD	0.49	0.07	3.67	0.07
106	TPN	0.09	0.03	0.40	0.04
	TPN+FAD	0.09	0.03	0.93	0.02
101	$CuCl_2$*	0.23	0.01	0.03	0.02
	$HgCl_2$*	0.23	0.01	0.02	0.00

* Asterisk indicates TPN, 1×10^{-5} M; $CuCl_2$, 12.5×10^{-3} M; $HgCl_2$, 5×10^{-4} M. All other concentrations, 7×10^{-4} M.

Pyridine nucleotide coenzymes. Both DPN and TPN stimulated the production of $C^{14}O_2$ from labeled glucose. In six experiments, TPN stimulated the production of 1-$C^{14}O_2$ by an average of 756 per cent and the production of 6-$C^{14}O_2$ by an average of 200 per cent. DPN, in three experiments, stimulated 1-$C^{14}O_2$ by an average of 1,233 per cent and 6-$C^{14}O_2$ by 800 per cent. In two experiments, the effects of full concentrations (7×10^{-4} M) of TPN and DPN were compared in the same homogenate. In one of these, the stimulatory effects of the coenzymes were essentially the same. In the second experiment, in which iodinations were also measured, TPN caused a greater increase in the production of 1-$C^{14}O_2$ than did DPN. In this experiment, iodinations were slightly stimulated by TPN and were unaffected by DPN.

The interaction of DPN with TPN at 0.1 its usual concentration (i.e., 7×10^{-5} M) was also evaluated in two experiments. In both, the lower concentration of TPN stimulated 1-$C^{14}O_2$ pro-

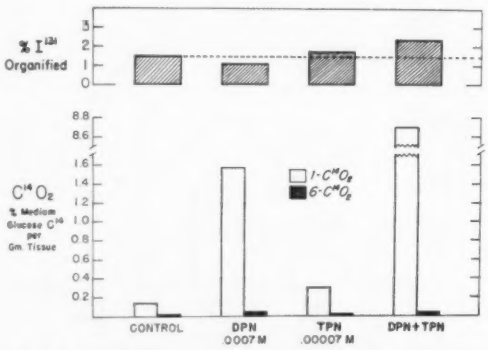

FIG. 12. THE EFFECTS OF TPN AND DPN, ALONE AND IN COMBINATION, ON ORGANIC IODINATIONS AND THE EVOLUTION OF $C^{14}O_2$ FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN A SHEEP THYROID HOMOGENATE.

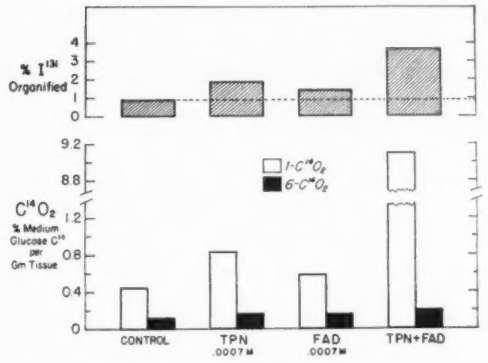

FIG. 13. THE EFFECTS OF TPN AND FAD, ALONE AND IN COMBINATION, ON ORGANIC IODINATIONS AND THE EVOLUTION OF $C^{14}O_2$ FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN A SHEEP THYROID HOMOGENATE.

duction only slightly as compared with the usual effect of the tenfold greater concentration of TPN. The generation of 6-$C^{14}O_2$ was also increased, but to a relatively slight extent. In one of these experiments, iodinations were also measured (Figure 12). These were slightly increased by DPN and markedly increased by the combination of coenzymes.

Flavin coenzymes. FAD increased the yield of 1-$C^{14}O_2$, but to a lesser extent than did TPN (Figure 13). The addition of FAD with TPN produced a greater stimulation of both $C^{14}O_2$ production and iodinations than could be accounted for

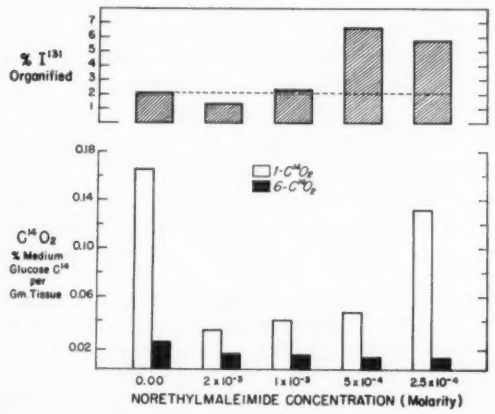

FIG. 14. THE EFFECTS OF NORETHYLMALEIMIDE ON ORGANIC IODINATIONS AND THE EVOLUTION OF $C^{14}O_2$ FROM GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN A HOMOGENATE OF SHEEP THYROID GLANDS.

TABLE X

The effect of thyrotropin (TSH) on the production of carbon dioxide from differentially labeled glucose by slices of sheep thyroid gland

Expt. no.	$C^{14}O_2$ production (% added glucose/g tissue)			
	Control		TSH	
	1-C^{14}	6-C^{14}	1-C^{14}	6-C^{14}
83	7.6	2.3	15.2	3.3
86a*	1.7	0.1	3.5	0.3
b	2.2	1.0	8.1	1.0
93	3.9	3.3	4.7	2.7
94	1.3	0.4	3.1	0.4
95	2.1	0.2	3.5	1.0
96	3.5	1.3	5.8	1.0
111	3.3	0.6	9.2	1.0

* Slices from same gland: a, incubated for 45 minutes; b, for 140 minutes.

by their individual effects. Other experiments confirmed the ability of FAD to enhance the stimulatory effect of TPN and revealed a similar action of FMN.

Methylene blue. In two experiments, the addition of MB to thyroid slices increased 1-$C^{14}O_2$ from an average of 1.68 to 15.46 per cent and 6-$C^{14}O_2$ from 0.27 to 1.00 per cent of added glucose-C^{14} per g of tissue.

Sulfhydryl inhibitors. NEM, 2×10^{-3} M, markedly inhibited the production of both 1- and 6-$C^{14}O_2$; at this concentration iodinations were also inhibited. With decreasing concentrations of NEM, the production of 1-$C^{14}O_2$ returned toward normal. In the case of 6-$C^{14}O_2$ a dose-response relationship was not apparent. As the evolution of 1-$C^{14}O_2$ approached normal values, a stimulation of iodinations became evident (Figure 14). Like NEM, $CuCl_2$ and $HgCl_2$ increased iodinations at concentrations which depressed the production of $C^{14}O_2$ (Table IX).

TSH (Table X). In seven experiments in which thyroid slices were incubated with TSH, yields of 1- and 6-$C^{14}O_2$ were increased by an average of 107 and 14 per cent, respectively. In three experiments in which these were measured, oxygen consumption and utilization of stable glucose were increased in TSH-supplemented flasks by an average of 15 and 102 per cent, respectively.

DISCUSSION

The present findings strongly suggest that the organification of iodide is linked to pathways of

carbohydrate metabolism in the thyroid gland. Ideally, this relationship would have been characterized in the intact animal. However, the difficulty of altering the metabolic environment of a single organ *in vivo* precluded this approach. Even the surviving slice system was considered unfavorable for these studies since, in slices, controlled changes in the intracellular concentration of metabolites could not be produced. Furthermore, since intact thyroid slices are capable of concentrating inorganic iodide, changes in organically-bound iodine resulting from alterations in the iodide-concentrating mechanism could not be differentiated from those due to changes in the rate of organic binding of intrathyroidal iodide. These difficulties were overcome by the use of a homogenate system. This abolished the iodide-concentrating mechanism and also permitted the direct introduction of the compounds under study. The whole homogenate, rather than specific subcellular fractions, was chosen for study in order to preserve, as far as possible, the metabolic interrelationships present in the intact cell, and since the primary objective of this study was an elucidation of the metabolic relationships of the iodinating system, it was considered of great importance to preserve glycolytic activity in the preparation. Because of its reported effectiveness in maintaining glycolysis in liver homogenates (5), a potassium-rich medium was employed. This medium was found to support both glucose metabolism and iodination in the thyroid homogenate.

Several differences between the metabolism of iodine in the thyroid homogenate and the whole gland, however, raise the question of whether the homogenate is a suitable medium in which to study physiological iodinations. First, as the present and previous studies have shown (12), iodinations in the homogenate do not proceed appreciably beyond the synthesis of MIT; even when the quantity of iodine organified was increased by stimulatory factors or by the addition of inorganic iodide, appreciable quantities of diiodotyrosine were not formed. Second, the protein in which MIT is formed is not thyroglobulin (13). Finally, both Unknown 2 of Taurog and co-workers and the iodinated front-running material are prominent products of iodination in the homogenate, but are not found in significant proportions in the intact gland (14). The presence of these unusual iodinated products in the homogenate may indicate the accumulation of iodinated intermediates which, in the intact gland, are too rapidly utilized to be evident. However, if the front material is indeed an iodinated lipid, it would be an unlikely precursor of thyroid hormone but, like MIT, would result from an oxidation of iodide. That the same basic process determined the rate of formation of both MIT and front material is also suggested by the findings that rates of formation of origin and front materials underwent qualitatively similar changes under the influence of stimulatory or inhibitory agents.[6] Thus, the evidence suggests that, within the homogenate, iodide is oxidized to a more reactive state which, in the face of structural disorganization, randomly iodinates available substrate.

In its inability to carry its iodinations beyond MIT, the thyroid homogenate resembles certain stages in the development of the fetal thyroid (15), certain neoplasms of the gland (16), and glands which have been frozen or stored (17). These, too, may show evidence of a preponderant or exclusive synthesis of MIT. Thus, the initial oxidation of iodide is probably a sturdy function resistant to cellular disruption or damage, and the homogenate system appears well suited to the study of this step in the intrathyroidal metabolism of iodine.

A pronounced variation was noted in the percentile iodinations achieved in unsupplemented samples of different homogenates. The reasons for this variability are not known, although several factors may have been contributory. Some variation in the elapsed time between the death of the animals and initiation of the experiments inevitably occurred. Pronounced differences in the size and vascularity of thyroid glands employed from day to day were not infrequent. These differences in gross appearance may have been re-

[6] In order to verify increases in iodinations, revealed by TCA-precipitation, chromatographic analysis of homogenates was performed at least once in the case of every stimulatory factor. Except for $HgCl_2$, all stimulatory factors produced proportionate increases in front and origin materials. Mercury produced a greater increase in front than in origin material. Factors stimulatory to iodinations did not cause the appearance of significant quantities of iodinated products not found in control homogenates.

flected in variations in the metabolic activity, in the rate of endogenous hormonal synthesis or in the concentration of inorganic iodide in such glands and in the homogenates to which they contributed. Therefore, in order to facilitate comparison of results obtained in replicate experiments, organic iodinations in the presence of modifying factors have been calculated not only as a fraction of added I^{131}, but also as a percentage of organic iodinations obtained in control vessels.

Endogenous inhibition of iodinations. In early experiments, thyroid homogenates were dialyzed in order to determine whether dialyzable factors were essential for the organification of iodide. On the contrary, percentile iodinations were considerably and consistently increased by dialysis. Since percentile iodinations in both fresh and dialyzed homogenates were decreased by the addition of iodide, it seemed likely that the stimulatory effects of dialysis might have been due at least in part to a loss of iodide from the homogenate. However, an indication that the increase in iodinations observed in dialyzed homogenates was not due solely to loss of iodide was afforded by the observation that, like dialysis, storage of homogenates at 4° C increased subsequent percentile iodinations, although to a lesser extent. Since the concentration of inorganic iodide, if anything, increases during storage in the cold (18), the increases in percentile iodinations achieved in the stored homogenate must have reflected an increase in chemical iodinations. This suggested that the homogenate contained an inhibitor which was labile to storage and perhaps dialyzable.

These characteristics of the endogenous inhibitor suggested that it might be a nonprotein, sulfhydryl-containing compound, such as glutathione. This tripeptide is present in many tissues (19), including the thyroid (20). It is dialyzable, autooxidizes readily, especially in the presence of traces of copper, and has been shown to inhibit iodinations in the thyroid homogenate system (21). Furthermore, previous workers have reported that copper increases organic iodinations in the homogenate system (21, 22). Although this effect has been ascribed to the participation of copper either as a cofactor for a thyroidal iodinase (21) or, nonphysiologically, as a direct oxidant of iodide (22), the present observations were consistent with the hypothesis that copper increased organic iodinations by decreasing the concentration of an endogenous sulfhydryl inhibitor of iodinations. This could result from either formation of mercaptides or catalysis of sulfhydryl autooxidation (11). This conclusion was supported by the finding that the stimulatory effects of copper were shared by both mercury and the even more nearly specific sulfhydryl inhibitor, NEM. Furthermore, since dialysis of the homogenate altered the response to these agents, rendering stimulatory concentrations either less so or frankly inhibitory, it seemed unlikely that the heavy metals could be acting as direct oxidants of iodide or cofactors for an iodinating enzyme. These observations also indicated that dialysis of homogenate did more than merely lower the concentration of inorganic iodide. Rather, dialysis appeared to have removed the endogenous inhibitor of iodinations which the sulfhydryl reagents themselves were thought to attack. Within this context, the inhibitory effects of high concentrations of sulfhydryl reagents in the fresh homogenate, as well as the inhibition of iodinations produced in dialyzed homogenates by lower concentrations which had previously been stimulatory, could be explained by the sulfhydryl dependency of several glycolytic enzymes (23) which apparently participate in the iodination mechanism. With high concentrations of NEM, this supposition was confirmed by demonstrating a pronounced decrease in the evolution of CO_2 from C^{14}-labeled glucose.

If the endogenous inhibitor were indeed GSH, then its effect on iodinations could be explained by certain well known reactions of this compound. Thus, GSH might act as a competitive substrate for an oxidant of iodide, such as H_2O_2 (11): $H_2O_2 + 2\ GSH \rightleftarrows GSSG + 2\ H_2O$. Alternatively, GSH might reduce an oxidized form of iodine, as in the following reaction: $2\ GSH + 2\ I^0 \rightleftarrows GSSG + 2\ H^+ + 2\ I^-$. Indeed, the facility with which the latter reaction proceeds *in vitro* permits its use in the measurement of GSH and other reducing substances in blood and other tissues (24).[7]

[7] This technique depends on the re-reduction by GSH and other reducing substances of iodine generated from iodide by potassium iodate. It appeared to afford a means of assessing the potential of substances within a thyroid homogenate to reduce iodine and to behave like the pos-

The greater inhibitory effect of GSH than of GSSG was consistent with the foregoing reactions, and indicated some limitation in the ability of the unsupplemented homogenate to reduce the oxidized form. This limitation was overcome by the addition of TPN. This converted the rather variable effect of GSSG to a consistent inhibition, an effect which was even more readily apparent when iodinations in the presence of TPN and GSSG were compared with those obtained with TPN alone. Enhancement by TPN of the GSSG-induced inhibition of iodinations can be ascribed to the rapid reduction of TPN within the homogenate, since TPNH is a coenzyme for glutathione reductase (25), an enzyme known to be present in the thyroid gland (26).

If GSH is indeed the endogenous inhibitor of iodinations,[8] the oxygen dependency of iodination rates may be due in part to the propensity of GSH to undergo oxidation under conditions of increasing oxygen tension. However, this alone cannot explain the absolute oxygen requirement of the iodinating system, since anoxia inhibited iodinations in homogenates from which the endogenous inhibitor had been removed by dialysis or by the addition of sulfhydryl reagents.

The role of carbohydrate metabolism. To define the relation between carbohydrate metabolism and organic iodinations in the thyroid homogenate, several methods were employed to alter the activity of pathways of glucose catabolism. Extensive studies of the effects of adding substrates were not performed. However, succinate, and possibly isocitrate and oxaloacetate, appeared to stimulate iodinations. In view of the proposed mechanism of iodination, to be discussed below, the stimulation produced by succinate is of particular interest, since succinate, unlike other carbohydrate intermediates, is directly oxidized by a flavin dehydrogenase (28). The lack of effect of other substrates is difficult to interpret; their endogenous concentration in the homogenate may not have imposed any rate-limitation. Sufficient experiments to determine the effects of adding substrates to dialyzed homogenates, in which endogenous substrates can be presumed to be depleted, were not performed.

It has been suggested that the hexose monophosphate (HMP) shunt and the Embden-Meyerhof glycolytic pathway can be selectively stimulated by the addition of pyridine nucleotide coenzymes specifically associated with the dehydrogenases of each pathway. Thus, it is thought that the addition of TPN increases the activity of the HMP shunt (29), while DPN stimulates glycolysis (5, 29). The ability of both TPN and DPN to increase iodinations in the homogenate system therefore implicated carbohydrate metabolism in the regulation of organic iodinations. However, the more consistent and greater stimulatory effect of TPN than of DPN suggested that the two coenzymes might stimulate iodinations by different mechanisms. It was interesting, therefore, to determine how the effectiveness of each coenzyme was related to its oxidation-reduction state. The effects of adding the oxidized and reduced forms could not be directly compared because of the rapidity with which coenzymes added to tissues are brought to oxidation-reduction equilibrium. By adding each coenzyme together with its specific reducing system (isocitrate-isocitrate dehydrogenase for TPN; ethyl alcohol-alcohol dehydrogenase for DPN), it was possible to maintain within the homogenate higher concentrations of reduced coenzyme than would have followed the addition of the coenzyme alone. Under these conditions, the stimulatory effectiveness of TPN was enhanced, while DPN became inhibitory. These findings afforded further evidence of a difference in the action of the two coenzymes on organic iodinations, in that TPN stimulated iodinations in its reduced form, while DPN was apparently effective only in its oxidized form.

As shown in Figure 7, however, there appeared to be a limit beyond which increasing the concentration of TPNH no longer increased iodinations. In this experiment, the stimulation of iodinations which followed the addition of TPN

tulated endogenous inhibitor of iodinations. Iodometric titrations revealed a virtually complete loss of reducing materials following dialysis of the homogenate or addition of stimulatory concentrations of $CuCl_2$. Partial loss of reducing materials occurred upon storage of homogenates.

[8] The evidence which suggests that GSH is the endogenous inhibitor of iodinations would also be consistent with the possibility that the effect of GSH is mediated in part or entirely by compounds such as ascorbic acid with which glutathione is in oxidation-reduction equilibrium (27). In the present experiments, ascorbic acid proved to be markedly inhibitory to iodinations in the homogenate system.

continued at a time when the concentration of TPNH was no longer increasing. However, the further addition of TPN, with the attainment of a new equilibrium at an even higher concentration of TPNH, was attended by no further stimulation of iodination. In the presence of excess TPNH, other factors apparently become rate-limiting for iodinations.

An explanation for the seemingly paradoxical inhibition of iodinations produced by the addition of lower concentrations of TPN was sought in the interaction between TPN and the endogenous inhibitor. Since exogenous GSSG was capable of changing the effect of added TPN from stimulation to inhibition, it seemed possible that the effect of TPN could be similarly modified by the endogenous inhibitor. Support for this hypothesis was obtained from the observation that either dialysis or addition of NEM not only changed the effect of the low concentration of TPN (7×10^{-5} M) from inhibition to stimulation, but also increased the stimulatory effect of full concentrations of the coenzyme (7×10^{-4} M). Thus, the relatively low concentration of endogenous inhibitor (presumably glutathione) induced inhibition in conjunction with low concentrations of added TPN, just as the high concentrations of added GSSG produced inhibition with full concentrations of added TPN.

Thus, the stimulation of organic iodinations produced by TPN is limited by the presence of an endogenous inhibitor which may be glutathione. This contrasts with the mechanism of action of the heavy metals and of NEM; their stimulatory effect is evident only in the presence of an endogenous inhibitor whose action they prevent. TPN is rapidly reduced in the homogenate, and the resulting TPNH has two opposing effects on iodination: a stimulatory action discussed in greater detail below, and an inhibitory action which is related to the reduction of endogenous GSSG. Increased organic iodinations ensue if, as when large concentrations of TPN are added, the stimulatory effect of TPNH outstrips the balancing inhibitory action. Under physiological circumstances, such an internally damped system might have considerable value in stabilizing the rate of organic iodinations during changes in metabolic activity within the thyroid gland.

Experiments with 1-C^{14}- and 6-C^{14}-labeled glucose were performed in order to gain further insight into the relation between carbohydrate metabolism and organic iodinations, to determine the pathways in the thyroid responsible for the reduction of TPN, and to elucidate the mechanism of action of other factors stimulatory to iodinations. The complexities of employing differentially labeled glucose to estimate the relative proportion or absolute amounts of glucose metabolized via the Embden-Meyerhof pathway *vis a vis* the HMP shunt have recently been reviewed (30). It is apparent that measurements of $C^{14}O_2$ production from glucose-1-C^{14} and glucose-6-C^{14} do not alone make such estimates possible. Nevertheless, a preponderant evolution of $C^{14}O_2$ from glucose-1-C^{14} over that from glucose-6-C^{14} provides evidence for the operation of the shunt. Furthermore, provided that proportionate re-formation of glucose from products of shunt activity remains unaltered, an increase in the evolution of $C^{14}O_2$, preponderantly from glucose-1-C^{14}, will indicate an increase in the activity of the HMP shunt and consequently in the rate of generation of TPNH. The present findings with labeled glucose have been employed and interpreted in that light.

The greater evolution of $C^{14}O_2$ from glucose-1-C^{14} than from glucose-6-C^{14} found during the present studies in both slices and homogenates of thyroid tissue indicated that the HMP shunt is operative in the thyroid gland.[9] The data further suggested that an acceleration of the HMP shunt, induced by the addition of TPN, was responsible for the reduction of at least a considerable proportion of the added coenzyme and that in the thyroid, as in other tissues, the availability of TPN is an important rate-limiting factor for this metabolic pathway.

The stimulatory effect of DPN on organic iodinations was more variable and less pronounced than that of TPN, suggesting that its action in the homogenate was more complex. Experiments in which iodine or glucose metabolism or both were measured indicated that this was indeed the

[9] Field and his co-workers have also presented evidence for the operation of the HMP shunt in thyroid slices (31). These workers were unable to demonstrate generation of $C^{14}O_2$ from labeled glucose in thyroid homogenates. This discrepancy with the present findings may have resulted from the difference in homogenization medium employed in the two studies.

case, and that the effect of DPN depended upon the concentration of TPN in the homogenate. Thus, in the presence of concentrations of exogenous TPN which inhibited iodinations when added alone, inhibitory or insignificant effects of DPN on organic iodinations were changed to stimulation and stimulatory effects were increased.

This synergism between the two pyridine nucleotides might possibly have been due to the action of a transhydrogenase (32), oxidizing DPNH and reducing TPN to its stimulatory form, TPNH. However, the stimulatory effect of DPN on iodinations was decreased or abolished by the concomitant addition of a DPN-reducing system. The converse would have been expected had a DPNH to TPN transhydrogenation been responsible for the stimulatory effect of DPN. Nevertheless, attempts were made to demonstrate such a transhydrogenase in thyroid homogenates. By a method analogous to that employed by Colowick, Kaplan, Neufeld and Ciotti (33), the reduction of TPN was assessed spectrophotometrically following addition of a low catalytic concentration of DPN and a DPN-reducing system (ethyl alcohol-alcohol dehydrogenase). No transfer of hydrogen from DPNH to TPN could be demonstrated by this method.

Since DPN also appeared to synergize the action of TPN on the HMP shunt, other interactions between the two pyridine nucleotides seemed possible. First, in the presence of an active TPNH to DPN transhydrogenase (33), added DPN might increase the activity of the HMP shunt by speeding regeneration of the hydrogen acceptor, TPN. However, if such a transhydrogenase were present, addition of DPN should tend to reduce the availability of TPNH and thereby decrease organic iodinations; the converse was actually the case. Furthermore, direct spectrophotometric assay by a technique analogous to that described above failed to reveal a TPNH to DPN transhydrogenase in thyroid homogenates.

A further possible mechanism for the effect of DPN involves the generation of TPN from DPN via a DPN-kinase (34). Partial conversion of DPN to TPN might explain the greater stimulation of iodinations achieved by TPN than DPN when added to homogenates in equal concentrations. The ability of DPN to reverse the inhibition of iodinations produced by 7×10^{-5} M TPN would also be consistent with this hypothesis. This explanation, however, is not consistent with the observation that in the presence of 7×10^{-4} M DPN, TPN in a concentration of 7×10^{-5} M produced a far greater stimulation of 1-$C^{14}O_2$ production than did 7×10^{-4} M TPN alone.

For these reasons, an alternative explanation, similar to that proposed by others to account for the synergistic action of TPN and DPN on hepatic synthesis of fatty acids (35) seemed more attractive. This hypothesis suggests that DPN increases the activity of the HMP shunt by accelerating the metabolism of 3-carbon products of the shunt which re-enter the Embden-Meyerhof pathway. Addition of DPN would thereby increase the generation of TPNH. In the presence of low endogenous concentrations of TPN, DPN might increase the generation of TPNH to the same extent as the addition of low (7×10^{-5} M) concentrations of TPN alone. Inhibition of iodinations would then result from an accelerated reduction of GSSG. If the endogenous concentration of TPN were higher or were increased by exogenous TPN, addition of DPN might facilitate the generation of sufficient TPNH so that its stimulatory effect on iodinations would become manifest. Thus, the influence of DPN on organic iodinations would be mediated through its effects on the generation of TPNH.

It seemed unlikely, however, that reduced TPN per se could be responsible for the oxidation of iodide which is requisite for organification. Rather, TPNH must serve as an intermediate in the generation of an agent capable of oxidizing iodide. The sensitivity of the iodinating system to inhibition by cyanide and its absolute oxygen dependency focused attention on the possibility that further hydrogen transfers along the cytochrome system might be required for the formation of the postulated oxidant. However, in the present experiments, in contrast to the results of other studies (36), addition of cytochrome C to the whole homogenate had little effect on organic iodinations. Antimycin A, which inhibits reduction of the cytochromes (37), also failed to inhibit iodinations (38), and in the present experiments was slightly stimulatory. This favored the hypothesis that the cytochromes did not function as part of the iodinating system. Methylene blue (MB) permits the direct transfer of electrons to oxygen, thereby

bypassing the cytochrome system. MB was therefore added to homogenates to compete with the cytochrome system as a hydrogen acceptor. The marked increase in the evolution of 1-$C^{14}O_2$ which followed the addition of MB indicated its ability to oxidize TPNH in the intact thyroid slice, as it has been thought to do in liver slices (39). Furthermore, the stimulatory effect of MB on iodinations in the homogenate and its synergism with added TPN in this regard not only seemed to rule out a necessary participation of the cytochromes in the iodinating system, but also suggested that hydrogen transfers from TPNH to MB might constitute a partial model of the physiological iodinating system.

Unlike TPNH, reduced MB is capable of undergoing auto-oxidation to yield H_2O_2 (40). For some years, it has been thought that thyroidal iodide is oxidized by H_2O_2 in a reaction catalyzed by a glandular peroxidase (41, 42). Although H_2O_2 has not itself been demonstrated in the thyroid, a halogen-peroxidase, apparently specific for iodide has recently been found (43) and a number of peroxidase inhibitors are known to be potent antithyroid agents (44). Furthermore, catalase has been shown to inhibit iodinations in both a soluble tyrosine-iodinating system derived from submaxillary gland (45) and in a thyroid mitochondrial-microsomal preparation (13). In the present experiments, catalase was moderately inhibitory of organic iodinations in whole thyroid homogenates. Finally, hydrogen peroxide has been shown to overcome the inhibitory effect of anoxia on iodinations in the tyrosine-iodinating system (45). It therefore seemed possible that the stimulation of iodinations induced by MB in the present experiments was due to its ability to generate H_2O_2 after reduction by TPNH.

In physiological systems, FAD-linked metalloproteins are the only known sources of H_2O_2. These aerobic dehydrogenases are inhibited by cyanide and are capable of being oxidized by MB. These properties suggested that, in the physiological iodinating system, a flavin enzyme might serve to accept hydrogen from TPNH and then produce H_2O_2 by auto-oxidation. For these reasons, the effects of added flavins in the thyroid homogenate were assessed. Although FAD alone induced only slight stimulation of organic iodinations and the evolution of $C^{14}O_2$ from labeled glucose, it acted synergistically with added TPN to produce a marked increase in iodinations and a profound increase in $C^{14}O_2$. Within the context of the H_2O_2 hypothesis, it appeared that FAD potentiated the action of TPN on the HMP shunt by maintaining the TPN in the oxidized state, while the resulting reduced FAD underwent auto-oxidation to form the oxidant of iodide, H_2O_2.

It was uncertain, however, that the effects of added FAD were indeed related to the function of the physiological iodinating system. Although previous workers had shown that iodinations in a thyroid mitochondrial-microsomal preparation could be increased by addition of flavins (38), this stimulatory effect was not abolished by anoxia. No explanation is apparent for the discrepancy between these and the present findings, in which FAD failed to stimulate iodinations in the absence of oxygen. More important, however, was the observation that in a tissue-free system, flavins could catalyze a light-dependent oxidation of iodide (46, 47) which could result in the iodination of added serum proteins. For these reasons, the effects of light and dark on organic iodinations in the present homogenate system, and on the actions of TPN and flavins therein, were assessed. The findings of earlier workers were confirmed, in that iodination of human serum albumin in a simple buffer medium could be effected by FAD in a reaction inhibited by exclusion of oxygen and light. However, TPN had no effect on the flavin-stimulated iodinations in this tissue-free system. In the thyroid homogenate, in contrast, exclusion of light had no effect on control iodinations, partially inhibited the increase in iodinations produced by FAD alone, but had no effect on the pronounced increase in iodinations which followed the addition of FAD with TPN. It seemed, therefore, that the added FAD was indeed participating in the physiological iodinating system of the homogenate, and that the energy for the flavin-stimulated oxidation of iodide, which was derived from incident light in the tissue-free system, was, in the thyroid homogenate, supplied by an endogenous metabolic source.

Most of these studies were performed with FAD. However, FMN was similarly effective. This lack of specificity with regard to coenzyme and the fact that the flavin-supplemented system seemed inefficient in the utilization of metabolic

energy, causing a proportionately much greater stimulation of 1-$C^{14}O_2$ production than of iodinations, makes it unlikely that free flavins are rate-controlling intermediates in the physiological iodinating system. Rather, it seems likely that in the thyroid, as elsewhere, the flavins are tightly bound to the enzymes with which they function.

Pronounced differences in the behavior of thyroid particulate preparations of differing nature have been noted. Thus, Tong and Chaikoff have reported that cytochrome C stimulated iodinations in the whole homogenate, but not in a mitochondrial-microsomal preparation (36), while the converse was true of FMN. Earlier, the stimulatory effect of flavins in a mitochondrial-microsomal preparation was reported to persist despite anoxia (38); in the present experiments a moderate stimulatory effect of flavins was obtained in whole homogenates and was distinctly inhibited by anoxia. Similarly, the present evidence that TPNH-linked dehydrogenations of the HMP shunt are regulatory to iodinations in the whole homogenate would presumably not apply to the mitochondrial-microsomal preparations. Here, however, mechanisms for the generation of reduced pyridine nucleotides and flavoproteins via the Krebs cycle could also lead to the formation of H_2O_2.

Because of these differences in the behavior of various broken cell preparations, experiments were undertaken to ascertain whether an increase in TPN-linked dehydrogenations could be demonstrated as a characteristic of physiological stimulation of the intact thyroid cell. For this purpose, TSH was employed to stimulate organic iodinations in thyroid slices. Relative effects on the evolution of $C^{14}O_2$ from 1-C^{14}- and 6-C^{14}-labeled glucose were again determined as an index of changes in the rate of generation of TPNH. While these studies were in progress, Field, Pastan, Johnson and Herring (31) reported the results of similar observations. The present results are entirely in accord with the findings of these workers, who observed that TSH increases the production of $C^{14}O_2$, preponderantly from 1-C^{14}- as compared with 6-C^{14}-labeled glucose, and who, like Freinkel (48), reported an increase in total glucose utilization under these conditions. These results indicate that TSH, a physiological stimulator of iodinations, is, in the intact thyroid cell, capable of enhancing those metabolic processes which, in the thyroid homogenate, provide energy for the oxidation of iodide. For reasons already noted, however, the findings do not permit conclusions as to whether TSH specifically or preponderantly stimulates the HMP shunt as compared to the Embden-Meyerhof pathway. Furthermore, they afford no evidence of how the evident increase in the turnover of TPNH is effected. Thus, TSH might accelerate the reduction of TPN by stimulating the HMP shunt, either specifically or as part of a general increase in glucose catabolism. Alternatively, TSH might stimulate hydrogen transfers within the physiological iodinating mechanism at some point distal to TPNH. The resulting increase in the generation of TPN from TPNH would increase the activity of the HMP shunt secondarily. The latter mode of action, analogous in some respects to that proposed for FAD, would seem more favorable to a stimulation of iodinations, since it would tend to decrease, rather than increase, the availability of TPNH for the reduction of GSSG. Finally, TSH might increase the availability of TPN by activating a DPN-kinase, if such is present in thyroid tissue (34). This explanation would be consistent with the observation that in the guinea pig thyroid, which is relatively inactive, the concentration of triphosphopyridine nucleotides (TPN + TPNH) is very low and is far exceeded by the concentration of diphosphopyridine nucleotides (DPN + DPNH) (49).

On the basis of the present findings, it is proposed that the energy for the thyroid organification of iodide is derived from glucose metabolism via TPN-linked dehydrogenations. TPNH is oxidized by a flavin enzyme which in turn reduces oxygen to form H_2O_2. The latter oxidizes iodide in a reaction catalyzed by a thyroidal peroxidase. DPN has a permissive action on the operation of the HMP shunt, but probably does not directly contribute hydrogen to the iodinating system. Dehydrogenations which reduce TPN are also capable of inhibiting iodinations by accelerating the reduction of GSSG. The latter reaction would serve as a "governor" on the acceleration of iodinations which would otherwise follow an increase in glucose catabolism.

Earlier observations have indicated that the maintenance of adequate intrathyroidal stores of

iodide for hormonal biogenesis depends upon phosphate bond energy derived from glucose catabolism (3, 4). The present observations afford evidence of a relation between glucose catabolism and the next step in hormonal biogenesis, the oxidation of iodide. The role of TPN-linked dehydrogenations in the latter reactions is of particular interest in view of recent indications that the synthesis of protein (50) and fat (35, 51) may be TPNH-dependent. Thus, the energy for increased hormonal biogenesis (accelerated synthesis of thyroglobin plus increased iodinations) and for its usual concomitant, glandular hyperplasia, may be derived via the same metabolic pathways. In this way, certain functional and structural effects of TSH on the thyroid gland could be mediated by an action upon a single metabolic site.

SUMMARY

The role of intermediary carbohydrate metabolism in regulating the rate of organic iodinations in thyroid homogenates has been investigated. Evidence was obtained for the presence of an endogenous inhibitor of iodinations whose activity was diminished or lost following storage, dialysis, or addition of sulfhydryl inhibitors.

Rates of organic iodination were regulated by TPN-linked dehydrogenations. Reduced TPN participated in both inhibitory and stimulatory interactions with the physiological iodinating system. The former appeared to result from an enhanced generation of the endogenous inhibitor, presumably reduced glutathione. The stimulatory interaction was synergized by flavin cofactors and methylene blue in reactions thought ultimately to yield hydrogen peroxide, an oxidant of iodide.

Dehydrogenations in the hexose monophosphate shunt appeared to be the principal source of reduced TPN. DPN and flavin cofactors acted synergistically with TPN in stimulating shunt activity and increasing generation of TPNH. In the intact thyroid cell, activity of the shunt was enhanced by TSH, a physiological stimulator of iodinations.

On the basis of these observations, an internally damped sequence of hydrogen transfers via TPN and a flavin enzyme is proposed as a means of utilizing energy from glucose metabolism in the oxidation of iodide.

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THE EFFECTS OF RETROGRADE PORTAL VENOUS FLOW FOLLOWING SIDE-TO-SIDE PORTACAVAL ANASTOMOSIS: A COMPARISON WITH END-TO-SIDE SHUNTS *

By JOHN F. MURRAY and DONALD G. MULDER with the technical assistance of LIANA NEBEL

(From the Department of Medicine and Surgery, University of California Medical Center, Los Angeles, Calif.)

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Reversal of blood flow in the portal vein has been demonstrated in a few patients with Laennec's cirrhosis (1, 2). This circulatory abnormality is probably uncommon and occurs only when the disease has distorted vascular pathways so that outflow resistance through the hepatic veins exceeds that existing through the portal venous channels. Retrograde flow in the hepatic branch of the portal vein following side-to-side portacaval anastomosis has been more consistently demonstrated in man (1, 2) and experimental animals (3). Reversal of flow in the portal vein, whether it occurs in an occasional patient with portal cirrhosis or, more commonly, following side-to-side portacaval anastomosis, implies that the entire inflow of blood to the liver is through the hepatic artery; this leaves two routes available for the egress of blood—the usual circulatory pathways out the hepatic veins and by retrograde flow out the portal vein. The hepatic artery is also the only blood supply to the liver following an end-to-side portacaval anastomosis; however, because no flow is possible from the liver out the ligated portal vein, all of the arterial inflow passes through the liver lobule and leaves via the hepatic veins. The important difference between end-to-side shunts on the one hand, and both portal cirrhosis and side-to-side anastomosis on the other, is that the increment of blood flowing out the portal vein would occur at the expense of circulation through the hepatic veins. This condition would be of no concern, or might even be advantageous, if the pathways between the hepatic artery and portal vein carried the blood through the hepatic sinusoids. However, if communications between these vessels occurred at the presinusoidal level, perfusion of the liver lobule might be impaired and cause hepatocellular ischemia.

The purposes of this study were twofold: first, to study some of the functional aspects of retrograde portal venous blood flow in dogs following surgical side-to-side portacaval anastomosis; and second, to compare the effects of side-to-side with end-to-side types of anastomoses in the otherwise normal dog.

MATERIALS

Successful experiments were performed on 26 mongrel dogs divided into three groups. Group 1 consisted of 13 dogs (nos. 1–13) studied from two different aspects designed to evaluate the effects of side-to-side portacaval anastomoses. In 8 splenectomized dogs (nos. 1–8) studied 1 to 12 months after a side-to-side shunt the experimental preparation shown in Figure 1 was used. Under pentobarbital anesthesia, cardiac catheters (no. 8 or 9F) were passed via both external jugular veins into a branch of the hepatic vein (HV) and the inferior vena cava (IVC). A laparotomy was performed, the anastomosis visualized, and under manual guidance the IVC catheter was placed 3 to 5 cm cephalad to the shunt. Another catheter (no. 6 or 7F), passed via the femoral vein and guided through the shunt, was directed toward the liver and left in the portal vein 4 to 5 cm from the liver (PV_H, the subscript refers to the hepatic side of the shunt). A polyethylene catheter (PD 90) was inserted into a distal mesenteric vein near the intestine and passed proximally until it could be felt in the portal vein 5 to 7 cm above the shunt (PV_G, the subscript refers to the gut side of the anastomosis). Finally, the femoral artery (FA) and vein (FV) were cannulated.

The direction of flow through the anastomosis in 6 dogs (nos. 1–6) was determined by regional indicator dilution studies. Indocyanine green (4) was injected into the FV, PV_H and PV_G catheters while blood was sampled from either the IVC or HV catheter with a Harvard constant speed withdrawal apparatus.[1] Time-

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[1] Dover, Mass.

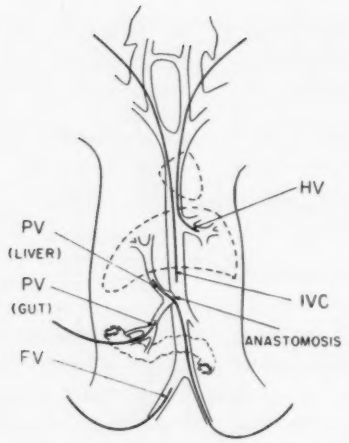

FIG. 1. SCHEMATIC REPRESENTATION OF AN EXPERIMENTAL ANIMAL AFTER A SIDE-TO-SIDE PORTACAVAL ANASTOMOSIS. The positions of the sampling and injecting catheters are shown. Abbreviations are given in the text.

concentration curves were detected with a Colson cuvet densitometer [2] and inscribed on a Varian G-10 recorder.[3]

After establishing that retrograde flow occurred in the PV_H segment of the portal vein, the experiment was amended to evaluate its effects on hepatic metabolism. In 5 dogs (nos. 4–8) a priming dose of 1 to 3 mg per kg of sulfobromophthalein (BSP) was followed by a sustaining infusion of 0.06 to 0.10 mg per kg per minute into the FV through a Bowman constant speed infusion pump.[4] After 30 minutes, two or three successive 10-minute simultaneous blood samples were obtained from the FA, HV and PV_H cannulas. Dog 13 was studied intact and then had a laparotomy to allow aspiration of blood from PV_H with a syringe so only single sample comparisons are reported. Plasma BSP concentration was determined in duplicate by the method of Gaebler (5). Heparinized blood was obtained anaerobically from the same sites and the PV_G cannula in Dogs 4–8, and from the FA and HV in Dogs 9–13 for determination of oxygen content by the manometric method of Van Slyke and Neill (6). Oxygen capacity was measured in the arterial sample; percentage saturation at all collection sites was related to the single capacity measurement after ensuring that hemoglobins and hematocrits were the same in all samples (if not, appropriate corrections were made).

Group 2 consisted of 7 dogs in each of which a splenectomy and end-to-side portacaval anastomosis were performed 1 to 12 months prior to final study. Oxygen and BSP determinations were the same as in group 1 animals with the exception that PV_H samples were unobtainable because the liver limb of the PV is obliterated in end-to-side shunts. Total hepatic blood flow was calculated by the method of Bradley, Ingelfinger, Bradley and Curry (7), using an assumed plasma volume of 90 ml per kg and the uncorrected arterial hematocrit. There were no problems regarding the direction of blood flow following end-to-side shunts so indicator dilution studies were not performed.

Group 3 consisted of 6 dogs in each of which no portacaval shunt had been performed. Each had a laparotomy so that a complete set of samples could be obtained as in group 2 dogs.

[2] Elyria, Ohio.

[3] Palo Alto, Calif.

[4] Brooklyn, N. Y.

RESULTS

Directional flow studies (group 1; Dogs 1–6). The proper position of the HV catheter was ascertained in each dog by injecting indocyanine green into the FV while withdrawing blood from the HV through the cuvet. If the catheter was not far enough into the HV, the slightest reflux of dye from the IVC could be easily detected by the prompt inscription of a curve with a sharp upstroke; a low, flat and much delayed curve, seen with a correctly placed catheter, indicated that the dye had made its appearance following passage through the lungs and had arrived at the liver via the systemic circulation and hepatic artery. Indocyanine green was injected into the portal vein on the intestinal side of the anastomosis (PV_G) while sampling from the HV. In no instance was dye recovered from the HV, indicating the complete diversion of the intestinal portal venous drainage into the IVC. This was confirmed by injecting dye through the PV_G cannula while

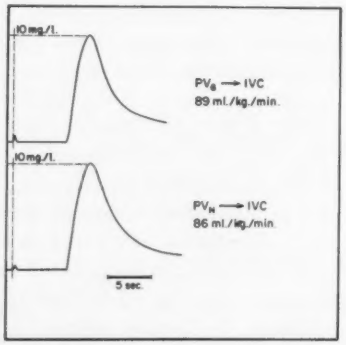

FIG. 2. SIMILARITY IN SUCCESSIVE INDICATOR DILUTION CURVES AS RECORDED FROM THE IVC CEPHALAD TO THE SHUNT OPENING AFTER PV_H AND PV_G INJECTIONS OF 1.25 MG INDOCYANINE GREEN. The catheter delay from the time of injection (the vertical spike) is 3.8 seconds. Abbreviations are given in the text.

sampling from the IVC cephalad to the anastomotic site; an early sharp dye curve (Figure 2) indicated blood flow through the shunt.

When dye was injected into the portal vein on the hepatic side of the anastomosis (PV_H), no early appearing dye was recorded at the HV, in all animals but two. In these two dogs large volumes of dye (5 ml instead of 1 ml) were injected rapidly, causing a transient rise in PV_H pressure. Using smaller (1 ml) volumes or a slower rate of injection resulted in successively lower curves until no dye was recorded at the HV, as shown in Figure 3. These curves demonstrate that the preferred direction of flow in the PV_H is from the liver out through the shunt to the IVC. When the pressure is raised in the PV_H the flow away from the liver can be temporarily reversed and blood will pass through to the HV. However, reversal of the retrograde flow in the PV_H was probably facilitated by the presence of the catheter passing through the shunt which narrows the orifice and increases resistance to flow out the anastomosis. It is conceivable that the flow of some dye from the PV_H into the liver might escape detection because extraction of indocyanine green will take place in a single passage through the sinusoids (8). However, curves were always promptly recorded from the IVC following PV_H injection and occasionally were quantitatively similar to curves obtained after PV_G injection (Figure 2). Barring problems from streamline blood flow (see below), this indicates that the same flow was being measured and should thereby account for all the dye injected.

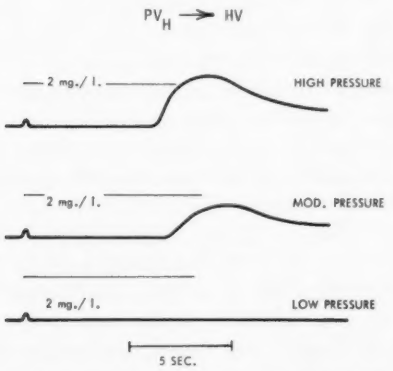

FIG. 3. INDICATOR DILUTION CURVES RECORDED FROM THE HV FOLLOWING PV_H INJECTION. The preferred retrograde portal venous flow (away from the HV) can be reversed by using high injection pressures. The amount of dye used and the catheter delay are the same as those given in Figure 2.

In most animals, however, sampling at the IVC following injections into the FV, PV_G and PV_H yielded grossly different curves. This means that either there is streamline flow and incomplete mixing of dye and blood or diversion of dye away from the sampling site. For reasons given above, it is felt that PV_G and PV_H blood flow is through the anastomosis and the curves should have similar areas; the failure to record identical curves indicates streamlining of blood flow. In two animals, dye was recovered in blood sampled from the IVC 2 to 3 cm caudad to the shunt opening following PV_H injection; this is best accounted for by a jet effect of the large blood flow from the liver traveling a few centimeters retrograde in the IVC.

Functional effectiveness of the retrograde portal venous flow (group 1). After the demonstration that retrograde flow occurred in the portal vein in dogs, studies were performed in ten dogs (nos. 4–13) to evaluate the functional significance of this blood flow. The results of oxygen and BSP analyses are presented in Tables I and II. There was greater extraction of BSP and oxygen in the blood flowing out the HV than out the PV_H. However, it was noteworthy that there was invariably some uptake of oxygen and BSP in PV_H, indicating that this blood had passed functioning hepatic cells and was being utilized. All five of the animals showed relatively the same division of function with evidence that the fraction of total hepatic inflow leaving the liver via the HV was being utilized to a greater degree than the outflow via PV_H. In order to decide if some of the results found in Dogs 4–8 were induced in part by the effects of the laparotomy which was performed to allow portal venous sampling, Dogs 9–13 were studied with only HV catheterizations and without abdominal surgery. No PV_H samples could be obtained, but the same large arterial-hepatic venous oxygen differences and BSP extractions were noted as in Dogs 4–8 that had had a laparotomy; therefore, the data on Dogs 4–8 were not considered to have been influenced by the collection methods.

Arterial oxygen saturation (FA_{O_2}). The mean FA_{O_2} for ten side-to-side, seven end-to-side, and

TABLE I

Oxygenation data in intact and laparotomized dogs after side-to-side portacaval anastomosis and splenectomy *

Dog no.	Wt	Hct	FA			HV		PV_H		PV_G		A-HV	A-PV_H
			Cap.	Cont.	Sat.	Cont.	Sat.	Cont.	Sat.	Cont.	Sat.		
	kg	%	*vol %*		%	*vol %*	%	*vol %*	%	*vol %*	%	*vol %*	
						A. Laparotomy							
4	22.8	40.0	18.0	16.2	90	6.4	35	13.1	73			9.8	3.1
5	17.0	39.2	17.5	16.0	91	7.0	40	14.0	80	10.2	58	9.0	2.0
6	20.4	37.8	17.3	15.4	89	8.8	51	11.8	68	13.2	76	6.6	3.6
7	20.0	37.5	16.8	15.4	92	5.0	30	11.5	68	12.4	74	10.4	3.9
8	17.8	46.2	21.4	18.6	87	7.0	33	14.7	69	12.9	60	11.6	3.9
						B. Intact							
9	17.1	34.5	16.0	14.6	91	6.6	41					8.0	
10	20.6	47.6	21.8	18.9	87	8.5	39					10.4	
11	15.7	35.3	15.6	13.4	86	5.8	37					7.6	
12	15.4	29.3	13.1	11.0	84	4.4	33					6.6	
13	17.7	44.6	21.1	19.5	92	8.5	40					11.0	
Mean		39.2			88.9		37.9		71.6		67.0	9.10	3.30
SEM		1.79			0.89		1.83		2.25		3.97	0.573	0.361

* Abbreviations: Hct, arterial hematocrit; FA, femoral artery; HV, hepatic vein; PV_H, portal vein, hepatic limb; PV_G, portal vein, gut limb; A-HV, arterial-hepatic vein oxygen difference; A-PV_H, arterial-portal vein (hepatic limb) oxygen difference; Cap., oxygen capacity; Cont., oxygen content; Sat., oxygen saturation; SEM, standard error of the mean.

six control dogs was 88.9, 90.0 and 88.8 per cent, respectively. These and the oxygen data given below are from single sample determinations in each dog. The lower than normal levels undoubtedly reflect the respiratory effects of the anesthesia and the duration of the experiment. No difference was encountered between the three groups of animals, as shown in Tables I and III.

TABLE II

Mean plasma sulfobromophthalein (BSP) values, two successive 10-minute samples, in group I dogs studied with laparotomy following side-to-side portacaval anastomosis *

Dog no.	FA	HV	PV_H	A-HV	A-PV_H	Cl_{BSP}
	mg %	*mg %*	*mg %*	%	%	*ml/min*
4	4.42	1.96	3.74	56	15	49
5	4.30	3.50	3.84	19	11	25
6	4.62	3.82	3.90	17	16	31
7	2.48	1.46	2.06	41	17	60
8	3.36	2.66	2.98	21	11	32
13†	3.36	2.42	2.96	28	12	41
Mean				29.3	13.7	39.7
SEM				6.24	1.29	5.33

* Abbreviations: A-HV extr., arterial-hepatic vein BSP extraction (FA-HV/FA × 100); A-PV_H extr., arterial-portal vein BSP extraction (FA-PV_H/FA × 100); Cl_{BSP}, BSP clearance (BSP removal rate/arterial BSP concentration). For other abbreviations, see footnote to Table I.
† Single samples only.

Hepatic vein oxygen saturation (HV_{O_2}). HV_{O_2} differed significantly between the three groups (Tables I and III). The mean values were: ten side-to-side dogs, 37.9 per cent (compared with controls $p < 0.001$, compared with end-to-side $p < 0.005$); seven end-to-side dogs, 55.6 per cent (compared with controls $p < 0.05$); and six controls, 66.2 per cent.

Arterial-hepatic venous oxygen difference ($FA–HV_{O_2}$). The virtually constant FA_{O_2} and the changing HV_{O_2} were reflected in a progressive widening of the $FA–HV_{O_2}$ between the six controls, seven end-to-side and ten side-to-side dogs; the mean values for these groups are 4.62, 6.24 and 9.10 vol per cent, respectively. These differ from one another at the same level of significance indicated above under HV_{O_2}.

Portal venous oxygen saturation (PV_{O_2}). The PV_{O_2} was higher in the six control animals (77.3 per cent) than PVG_{O_2} in five side-to-side dogs (67.1 per cent) and PV_{O_2} in four end-to-side dogs (72.0 per cent). The differences between these values were not statistically significant.

Hematocrits. A wide variation in hematocrits within all three groups was encountered (Tables I and II). The mean values in the ten side-to-side dogs (39.2 mm) and seven end-to-side dogs

TABLE III

Oxygenation data from end-to-side anastomosis and control animals *

Dog no.	Wt	Hct	FA			HV		PV		A-HV
			Cap.	Cont.	Sat.	Cont.	Sat.	Cont.	Sat.	
	kg	*%*	*vol %*		*%*	*vol %*	*%*	*vol %*	*%*	*vol %*
Group 2: End-to-side portacaval anastomosis										
14	24.1	45.7	20.9	19.5	93	14.9	71	16.8	80	4.6
15	19.8	39.6	18.7	16.2	86	10.5	56	13.3	71	5.7
16	21.7	46.2	22.2	19.5	88	14.6	65	16.3	73	4.9
17	18.0	47.7	21.9	19.5	89	11.5	52	13.9	64	8.0
18	17.5	27.5	13.0	11.3	87	3.4	26			7.9
19	18.7	43.6	19.8	18.2	92	10.6	53			7.8
20	16.7	38.0	16.8	15.9	95	11.1	66			4.8
Mean		41.2			90.0		55.6		72.0	6.24
SEM		2.65			1.24		5.58		2.58	0.596
Group 3: Control										
21	17.8	54.8	24.6	22.2	90	17.1	70	20.7	84	5.1
22	17.7	42.0	17.9	15.2	85	9.7	54	10.4	58	5.5
23	21.1	46.3	21.0	19.3	92	16.4	78	18.4	87	2.9
24	21.0	40.8	19.1	16.7	88	12.1	63	15.6	82	4.7
25	15.1	48.6	21.9	20.6	94	16.1	74	17.0	78	4.5
26	15.1	42.5	19.2	16.2	84	11.2	58	14.1	75	5.0
Mean		45.8			88.8		66.2		77.3	4.62
SEM		2.15			1.59		3.71		4.27	0.373

* The abbreviations are the same as noted in Table I. The three animals in group 2 without PV data did not have laparotomies.

(41.2 mm) were both lower than the mean value for the six controls (45.8 mm), but only the side-to-side difference was significant ($p < 0.05$).

Sulfobromophthalein studies. Considerable variation occurred in arterial plasma BSP levels when a constant prime (3 mg per kg) and sustaining infusion (0.10 mg per kg per minute) were used. Values in both groups of shunted animals, especially side-to-side dogs, tended to be higher than the controls, so eventually less BSP was given to these animals (1 mg per kg prime and 0.06 mg per kg per minute sustaining). All animals receiving BSP had an extraction greater than 10 per cent at plasma levels above 1 mg per 100 ml. The difference in arterial values precludes comparison of BSP extraction ratios. Mean plasma BSP clearance values (removal rate/arterial concentration) were 39.7 ml per minute for side-to-side, 61.4 ml per minute for end-to-end, and 81.2 ml per minute for control animals (Tables II and IV). Although there is a statistically significant difference between end-to-side and side-to-side dogs ($p < 0.05$), part of this discrepancy may be due to the higher arterial plasma BSP concentrations in the side-to-side animals and therefore does not necessarily reflect reduced hepatic func-

TABLE IV

Mean plasma sulfobromophthalein (BSP) values, two successive 10-minute samples, in end-to-side and control dogs with resultant estimated hepatic blood flow, percentage extraction and BSP clearance *

Dog no.	FA	HV	EHBF	EHBF	A-HV	Cl_{BSP}
	mg %		*ml/min*	*ml/kg/min*	*%*	*ml/min*
Group 2: End-to-side portacaval anastomosis						
14	2.60	1.68	369	15.3	35	68
15	3.80	2.50	293	14.8	34	55
16	2.14	1.32	213	9.8	38	44
17	1.64	1.00	227	12.6	39	44
18†	1.44	0.80	278	15.9	44	78
19†	3.31	2.68	509	27.2	19	51
20†	0.90	0.56	424	25.4	38	90
Mean				13.13		61.4
SEM				2.460		6.14
Group 3: Control						
21	2.26	1.22	404	22.7	46	84
22	2.04	1.38	428	24.2	32	74
23	1.02	0.64	580	27.5	37	110
24	2.00	1.31	479	22.8	35	92
25	1.42	0.84	350	23.2	41	70
26	1.48	0.78	269	17.8	47	57
Mean				23.03		81.2
SEM				1.277		7.57

* EHBF, estimated hepatic blood flow. For other abbreviations, see Tables I and II.
† Dogs studied without laparotomy.

tion. Total estimated hepatic blood flow (EHBF) could be calculated for the six controls and seven end-to-side dogs (Table IV); however, EHBF could not be measured in side-to-side dogs from available data because it is not known what fraction of the total amount of BSP arriving at the liver is distributed to the two exit pathways. There was a significant difference in mean EHBF between the six control dogs (23.0 ml per kg per minute) and the four laparotomized end-to-side dogs (Dogs 14–17, 13.1 ml per kg per minute). The control EHBF values are lower than those commonly encountered in the laboratory (mean, 39 ml per kg per minute). Part of this variation may be from the splenectomy and part due to the abdominal procedure; evidence for the latter is shown by the three end-to-side dogs (nos. 19–21) studied without laparotomies that had higher EHBF values than the other animals in their group (Table IV).

Other results. Barring technical difficulties from the shunt procedure itself, more problems were experienced in the postoperative management of side-to-side then end-to-side dogs; side-to-side animals recovered from surgery slowly, ate poorly and often lost weight. Although we have maintained side-to-side dogs in apparent good health for more than a year, six of them died prior to study for unexplained reasons. Only two end-to-side animals died under similar circumstances. Liver function studies were not done, but a lower BSP clearance was found in side-to-side dogs. The more severe anemia, intolerance of subsequent procedures, and fragility of the animals, suggest a greater hepatic disturbance induced by the side-to-side than end-to-side types of anastomosis.

DISCUSSION

This study substantiates previous observations that retrograde flow in the PV_H occurs following side-to-side portacaval anastomosis (1–3). The original investigations, in patients with portal cirrhosis at or shortly after side-to-side anastomosis, indicated that retrograde portal venous flow occurred, but its quantitative and metabolic aspects were not measured (1, 2). Long and Lombardo (3), using direct measures of flow in normal dogs following side-to-side shunts, revealed that outflow through the PV_H accounted for 70 per cent of the hepatic arterial inflow; however, the functional effectiveness of the portal venous circulation was not analyzed. The large volume of blood flowing retrograde in the PV under these conditions is readily explained by the creation of a new pathway to the IVC offering less resistance than does flow through the usual channels via the HV. When the PV is available as an outflow vessel, hepatic arterial blood, after arriving in the liver, may leave through two routes with different resistances to flow: *1*) through the sinusoids and the hepatic veins, and *2*) through the lower resistance communications to the PV; the latter pathway is preferentially utilized in the dog.

The principal purpose of the present study was to determine if the blood flowing retrograde in the PV_H had been effectively utilized by the liver. The results show clearly that there was invariably some extraction of oxygen and of sulfobromophthalein in the portal venous effluent. The low PV_H extractions might reflect either poor tissue uptake or high blood flow; the data do not distinguish between these possibilities. However, the hepatic venous outflow was invariably more efficiently utilized than was blood leaving through the PV_H. It is of interest that evidence of appreciable function was demonstrated in the portal venous outflow and it can be concluded that at least part of this blood passed by parenchymal liver cells.

In the normal dog following side-to-side portacaval anastomosis, the volume-capacity of existing presinusoidal arterial-portal venous communications would determine how much blood reaches the sinusoids. Although ample anastomoses between hepatic arterial and portal venous channels have been demonstrated, their precise location(s) remains controversial. It was felt by Mall (9) and by Olds and Stafford (10) that the communications occurred within the liver lobule at the periphery of the sinusoids. However, subsequent injection and transillumination studies have revealed a more liberal network at the presinusoidal level (11, 12). Figure 4 shows a schematic representation of the microanatomy of the hepatic lobule. In the normal mammal, hepatic arterial blood may communicate with the portal venous circulation within the sinusoids and by presinusoidal connections with interlobular branches of the PV. The many anastomoses be-

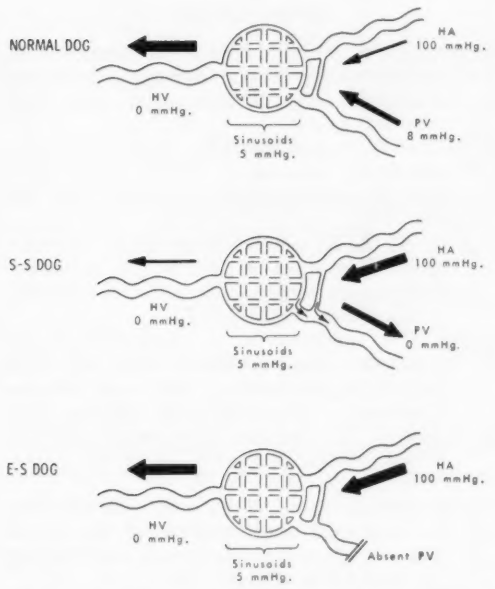

FIG. 4. SCHEMATIC REPRESENTATION OF INTRAHEPATIC BLOOD FLOW IN THE NORMAL DOG AND AFTER SIDE-TO-SIDE OR END-TO-SIDE PORTACAVAL ANASTOMOSIS. The thickness of the arrows indicates the relative magnitude of flow. The significance of the alterations is discussed in the text.

tween the HA and PV before or at the periphery of the sinusoids would offer immediate access of hepatic arterial blood to the portal venous circulation. The appreciable evidence of hepatocellular activity in the blood flowing out the PV is best explained by assuming that the interlobular hepatic arterial-portal venous communications cannot accommodate the entire inflow and that some arterial blood arrives at the liver lobule. Once this occurs, Elias (13) demonstrated that even though branches of the HA and PV meet at the periphery of the hepatic sinusoids, the inflows are separated by a streaming effect within the arterial circulation which serves to force blood toward the center of the lobule. In the same paper Elias (13) reported, and later strongly emphasized (14), arterial branches that open into the sinusoids as far into the lobule as two-thirds of the distance to the central vein. Both the "morphologically peripheral, yet physiologically central, arterial supply" (13) and the intralobular arterial communications enable a portion of the arterial blood to flow through the sinusoids to the HV and the remainder of the lobular inflow to depart via the PV where it joins with the presinusoidal "shunted" portion. There is no low resistance pathway to the IVC following end-to-side anastomosis; therefore, thorough perfusion of the lobule is maintained (Figure 4).

Without knowing the exact amount of blood distributed to the two exit pathways, it is impossible to state whether total liver function is impaired. It is possible that the excessive outflow through the PV_H occurs at the expense of adequate circulation to the center of the liver lobule. The very low HV_{O_2} indicates poor perfusion, hence large oxygen and BSP extraction, of the cells surrounding the central vein. Although the amount of ischemia necessary to interfere with hepatic cellular metabolism is unknown, it seems likely that optimal activity will be jeopardized in the oxygen environment existing after a side-to-side shunt. Poor hepatocellular function would explain why animals with side-to-side shunts are more anemic, have lower BSP clearances and clinically are more fragile than animals with end-to-side shunts.

A comparable marked reduction in HV_{O_2} was not noted in Warren and Muller's (2) studies on patients with liver disease after side-to-side anastomosis. Our own studies (15) in three patients tended to confirm these observations and, in addition, in two of our patients there was greater extraction of BSP and oxygen evident in the PV_H than in the HV outflow. However, one of the subjects had an A-HV_{O_2} difference of 9.9 vol per cent and has not done well clinically following surgery; this may be due to progression of his underlying liver disease or may reflect inadequate sinusoidal perfusion following the shunting procedure. These reports (2, 15) indicate variations within the available patient data, and usually a contrast is found between the findings following side-to-side anastomosis in humans with liver disease and in normal dogs. The most likely explanation for these data lies within the distorted parenchymal architecture of Laennec's cirrhosis, which can affect the portal venous radicles or sinusoids to a different degree. Obstructive scarring either in branches of the portal vein or in the sinusoids would impede outflow through one channel and facilitate it through the other. It seems reasonable to conclude that, although most patients with Laennec's cirrhosis are benefited by a side-to-side anastomosis, the previously nor-

mal dog suffers some impairment of adequate perfusion of the central portion of the liver lobule with resulting liabilities.

SUMMARY

Hemodynamic and metabolic studies were performed on 13 dogs following side-to-side portacaval anastomosis. Directional flow studies indicated that the proximal portal vein no longer carried blood toward the liver but served as an outflow vessel. Available evidence indicates that hepatic arterial inflow communicates extensively with portal venous blood by presinusoidal and intralobular anastomoses; following a side-to-side shunt these vessels afford a low resistance pathway to the inferior vena cava which bypasses the hepatic veins.

The metabolic effectiveness of the outflow in the portal vein was compared with that in the hepatic veins. There was invariably uptake of oxygen and sulfobromophthalein (BSP) in the portal venous effluent; however, in no instance was the evidence of metabolic efficiency as great in the portal vein as it was in the hepatic veins. Moreover, it appeared that retrograde flow out the portal vein occurred at the expense of adequate perfusion of the center of the liver lobule. The resultant low hepatic venous oxygen content reflected central lobular ischemia which could impair hepatocellular function; this mechanism was felt to be responsible for the lower BSP clearance, greater anemia and poorer survival following side-to-side shunts when compared with end-to-side anastomoses.

Findings from comparable studies in human patients with liver cirrhosis and side-to-side shunt are usually different from those in the dog. It is concluded that portacaval shunts in otherwise normal dogs do not accurately reflect the consequences of similar anastomoses in patients with Laennec's cirrhosis.

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COMPARISON OF EFFECTS OF PROTAMINE AND POLYBRENE, WITH SPECIAL EMPHASIS ON THE FACTOR VIII (ANTI-HEMOPHILIC GLOBULIN) DEFICIENCY INDUCED *

By HERBERT A. PERKINS, GEORGE HARKINS, FRANK GERBODE, MARY R. ROLFS AND D. J. ACRA

(From the Research Laboratory of the Irwin Memorial Blood Bank of the San Francisco Medical Society, and the Surgical Research Laboratory of the Presbyterian Medical Center, San Francisco, Calif.)

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Successful achievement of extracorporeal circulation requires that blood coagulability be temporarily interrupted during perfusion and afterward restored to normal. Heparin is accepted as the drug of choice in rendering the blood incoagulable, but there are two drugs, protamine and Polybrene (hexadimethrine bromide), available for effective *in vivo* neutralization of heparin, and controversy exists as to which of these should be used. In an effort to determine which of these heparin antagonists is superior, comparative studies on protamine and Polybrene were carried out to evaluate heparin neutralization, physiological and hematological effects, and such other matters as may be of practical importance.

In the course of these studies an unexpected fact became apparent. The intravenous injection of protamine or Polybrene resulted in a coagulation factor deficiency, persisting long after the temporary anticoagulant effect of the heparin neutralizer had disappeared. It was possible to demonstrate that a striking loss of Factor VIII (antihemophilic globulin) had been induced.

METHODS

Adult mongrel dogs were used throughout the study, and all animals were anesthetized with intravenous Nembutal (pentobarbital), 30 mg per kg. A measured dose of protamine [1] or Polybrene [2] (up to levels of 5 mg per kg) was rapidly injected into the inferior vena cava through a polyethylene catheter in the femoral vein and the tubing immediately flushed with 10 ml of normal saline. All samples were taken in a siliconized syringe from a catheter inserted into the femoral artery with its tip in the aorta. Prior to each sampling the tubing was first cleared of stagnant blood by removal of 10 ml of blood.

In experiments that did not require repeated withdrawal of blood samples, the two drugs were tested 8 weeks apart in the same animal under identical conditions, thereby allowing each animal to serve as its own control. In all other experiments the two were tested simultaneously in different animals.

Pulse and blood pressure recordings were made through a strain gauge connected to a femoral arterial catheter. Respirations were charted by means of a spirometer connected to a cuffed endotracheal tube. Standard limb lead electrocardiograms were taken.

Protamine and Polybrene levels were measured by heparin titrations, which were performed in some experiments by adding 1 ml of blood from the syringe to each of a row of 12 × 75 mm glass tubes in a water bath at 37° C. Each tube contained 0.1 ml of heparin in amounts of 0 to 50 μg at increments of 5 μg. Each tube was immediately capped with Parafilm and inverted twice to mix the contents. They were observed for clotting at intervals of 1 minute, the endpoint being a clot which remained solid on tipping the tube past horizontal and tapping it gently against the side of the water bath. The neutralization point was taken as the tube with the greatest amount of heparin in which the shortest clotting time occurred.

In other experiments the titrations were done on plasma from blood collected in 0.1 vol of 3.8 per cent sodium citrate. Each tube contained 0.1 ml of heparin in concentrations of 0 to 10 μg at increments of 1 μg plus 0.1 ml of the plasma to be tested. Then 0.1 ml of 0.025 M calcium chloride was added in rapid succession to each tube, they were mixed, and observed for clotting at intervals of one-half minute. Repeated titrations on the same sample over a period of 6 hours gave the same results.

Lee-White clotting times were done by placing 2 ml of blood in each of three 12 × 75 mm Pyrex tubes in the water bath. The endpoint was taken as the time at which the third tube could be tipped past horizontal without displacing the clot.

Platelet counts were performed on 5-ml blood samples collected in a 15 ml polyethylene vial containing 0.2 ml of 10 per cent disodium ethylenediamine tetraacetic

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[1] One per cent solution, Lilly.

[2] One per cent solution, Abbott.

acid (EDTA). The Brecher-Cronkite phase technique was used (3).

Plasma prothrombin times were determined on blood collected in 0.1 vol 3.8 per cent sodium citrate by the methods of Quick (4) and of Ware and Stragnell (5). True prothrombin assays were done by a one-stage technique (6). Prothrombin times on serum were determined after incubation of a 2 ml clot in a 12 × 75 mm Pyrex tube for 1 hour at 37° C. Consumption of prothrombin was stopped at that point by adding 0.9 ml of serum to 0.1 ml 3.8 per cent sodium citrate. The technique of Ware and Stragnell was used.

Cephalin times were done on citrated plasma by adding 0.1 ml optimal dilution of cephalin, prepared by the technique of Bell and Alton (7), and 0.1 ml of 0.025 M calcium chloride to 0.1 ml plasma. The plasma was glass-activated by incubating 0.1 ml in a new 12 × 75 mm Pyrex tube for 3 minutes at 37° C before performing the test.

Fibrinogen levels were measured in the EDTA plasma by the technique of Ratnoff and Menzie (8). *Thromboplastin screening tests* were done by the technique of Hicks and Pitney (9), using the Bell and Alton cephalin. *Thromboplastin generation tests* were done by the technique of Biggs and Macfarlane (10). Cephalin at optimal dilution was used as a platelet substitute except for experiments where the platelets themselves were being tested. Dilutions of the serum and the absorbed plasma reagents were made as outlined in the Results section.

Factor V levels were assayed by the technique of Borchgrevink, Pool and Stormorken (11) and also with the use of aged oxalate plasma as substrate (12). In this and all other clotting factor assays, the values are reported as percentage activity related to the control value before the experiment began.

Factor VIII (antihemophilic globulin) was assayed by the technique of Bergna (13) with the following modifications: Serum from clotted whole beef blood was used as a source of Factor IX, Factor X and Factor V. Outdated blood bank plasma from blood collected in acid citrate dextrose solution was used as the substrate on which the amount of plasma thromboplastin generated was assayed. In all assays, appropriate dilutions of plasma were made so that clotting times of the test plasmas would fall in the central part of the curve provided by dilutions of the control plasma (1 : 500, 1 : 1,000, 1 : 2,000, and 1 : 4,000). In one experiment, Factor VIII was measured by the cephalin time technique of Langdell, Wagner and Brinkhous (14) using plasma from a proven classical hemophiliac as substrate.

RESULTS

Physiological effect. Table I presents the changes in blood pressure caused by the two drugs at doses of 3 and 5 mg per kg, both with and without prior heparinization. Analysis of these data reveals that the rapid injection of either drug induces a profound hypotension, that the reduced blood pressure persists for several minutes, and that the degree of shock is not influenced by the presence or absence of heparin. Although these effects were more severe and more consistent with protamine than with Polybrene, the differences were small and not statistically significant.

The character of the changes in blood pressure was the same with both drugs. Alterations in pulse were also identical. There was a brief tachycardia followed by a protracted bradycardia. Electrocardiographic tracings in these animals revealed no conduction abnormality with either drug.

Both drugs were capable of producing a pronounced slowing in respiration, at times amounting to apnea (Figure 1), followed by prolonged tachypnea. Again these changes were slightly

TABLE I

Comparison of Polybrene and protamine in blood pressure depressing effects

Expt. no.	Dog no.	Drug	Dose	Heparinized	Average mean BP before injection	Lowest mean BP after injection	Maximum drop in mean BP	Drop in BP	Duration of significant BP depression
			mg/kg	*3 mg/kg*	*mm Hg*	*mm Hg*	*mm Hg*	*%*	*min*
1	1	Polybrene	3	Yes	135	55	80	60	4
2	2	Polybrene	3	Yes	165	90	75	45	7
3	1	Protamine	3	Yes	125	45	80	64	2
4	2	Protamine	3	Yes	128	63	65	51	1
5	3	Polybrene	5	Yes	105	105	0	0	
6	4	Polybrene	5	Yes	130	20	110	85	3
7	3	Protamine	5	Yes	135	55	80	60	26
8	4	Protamine	5	Yes	150	50	100	67	23
9	5	Polybrene	3	No	115	50	65	57	13
10	6	Polybrene	3	No	120	50	70	58	5
11	5	Protamine	3	No	110	40	70	64	5
12	6	Protamine	3	No	115	80	35	30	10
13	7	Polybrene	5	No	133	108	25	19	3
14	8	Polybrene	5	No	120	20	100	83	17
15	7	Protamine	5	No	165	55	110	67	6
16	8	Protamine	5	No	140	30	110	79	30

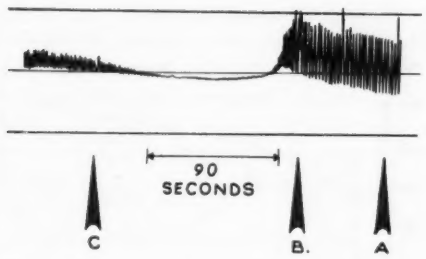

FIG. 1. RESPIRATORY CHANGES INDUCED BY PROTAMINE. A) Beginning of control period; B) injection of protamine; C) recovery period.

more pronounced with protamine but the difference is not significant.

Heparin-neutralizing effect. The comparative ability of protamine and Polybrene to neutralize heparin in blood *in vitro* was determined by adding human blood containing 25 μg (2.5 U) of heparin per ml to parallel rows of tubes containing protamine and Polybrene in 0.1 ml saline in quantities from 0 to 50 μg. The results of five such experiments are shown in Figure 2. Two points of significance stand out. Fifteen μg of Polybrene neutralizes 25 μg of heparin, whereas 25 to 35 μg of protamine is required. The results with Polybrene are definitely more consistent.

The disappearance of injected protamine and Polybrene from the circulating blood occurred at a rapid rate, as measured by ability of the blood to neutralize heparin in the titration test (Table II). As one would expect, the larger the dose given the higher the levels achieved, and the longer detectable amounts persisted. A second injection of Polybrene given 1 hour after the first (when all traces were gone from the blood) resulted in far greater concentrations. The apparently higher levels of Polybrene compared with those of protamine are very likely due to the fact, shown above, that it takes less of the former

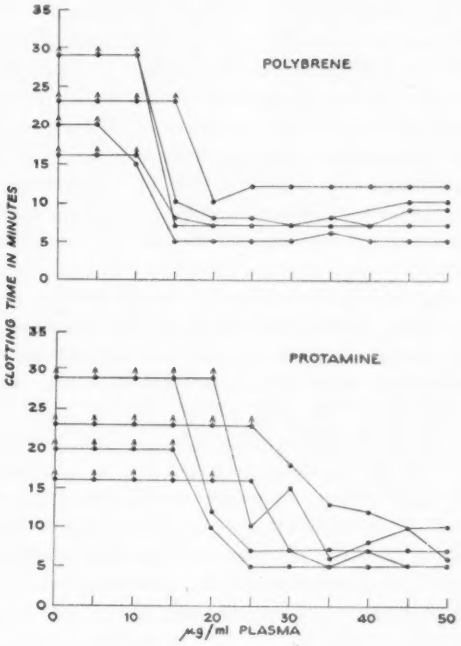

FIG. 2. CLOTTING TIMES OF HEPARINIZED BLOOD IN PRESENCE OF VARIOUS QUANTITIES OF POLYBRENE AND PROTAMINE. Arrows indicate that clotting time was longer than points recorded.

TABLE II

Mean protamine and Polybrene levels after injection *

Heparin neutralizer	Whole blood or plasma	Dose	No. of expts.	Minutes after injection 2	5	10	20
		mg/kg		μg			μg
1 Polybrene	Blood	5	3	25	8	2	0
2 Protamine	Blood	5	3	25	10	0	0
3 Polybrene	Blood	3	2	20	5	0	0
4 Protamine	Blood	3	3	8	0	0	0
5 Polybrene	Blood	2	2	5	0	0	0
6 Protamine	Blood	2	3	5	0	0	0
7 Polybrene	Plasma	5	9	23 ± 6†	13 ± 5	8 ± 5	2 ± 3
8 Polybrene	Plasma	5‡	5	48 ± 14	34 ± 9	23 ± 8	16 ± 5

* The figures quoted in the table represent the maximum amount of heparin (μg) neutralized by 1 ml of blood or plasma.

† Standard deviation.

‡ Second injection 60 minutes after the first one.

to neutralize a given amount of heparin. The close similarity in the amount of heparin neutralized by 1 ml of whole blood (line 1, Table II) and by 1 ml of plasma (line 7, Table II) at any given time after the injection of Polybrene is very interesting. For present purposes it is of importance only that the rate of disappearance was the same by either technique. It seems worth commenting, however, that the use of plasma has a number of advantages: the test can be done at leisure, smaller amounts of blood are needed, and the added antiheparin effect of red blood cells and platelets is eliminated. The elimination of antiheparin effect of the cellular elements may explain why the antiheparin effect of 1 ml of plasma is no greater than that of 1 ml of blood (containing approximately 0.5 ml of plasma). The close agreement of the antiheparin effect of 1 ml of whole blood containing Polybrene with that of 1 ml of plasma has been confirmed by simultaneous titrations on the same blood sample. It has not been possible to detect Polybrene in the red cells.

Effect on blood coagulation. The results of the injections on the clotting time of whole blood are shown in Figure 3. Despite the marked variability in the response of individual dogs, a consistent pattern emerged. One minute after injection there was a marked shortening of the clotting time while at 2 minutes it was markedly prolonged over control values. There was a rough correlation between the degree of effect and the dose of heparin antagonist administered. Prior injection of heparin lessened the effect. Many of the 2-minute samples never clotted despite the fact that addition of 0.1 vol of undiluted (1 per cent) Polybrene or protamine *in vitro* to blood could not prolong its clotting time to that extent. This was the first clue that these heparin neutralizers were doing more to the blood coagulation system than could be explained by their known weak anticoagulant effect. There was no significant difference between the two heparin antagonists in these results.

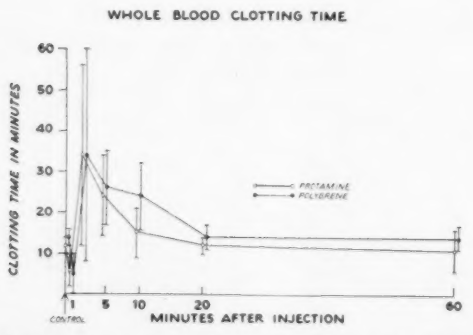

FIG. 3. WHOLE BLOOD CLOTTING TIME. In this and all subsequent figures the vertical lines represent 1 standard deviation.

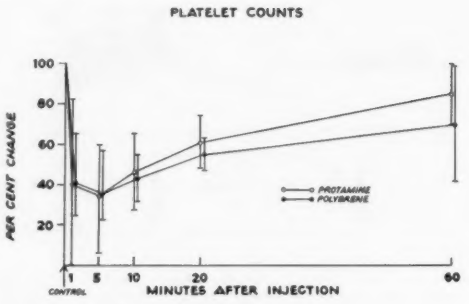

FIG. 4. PLATELET COUNTS.

Platelet counts showed that both drugs caused an immediate profound thrombocytopenic effect with a slow return to normal levels (Figure 4). There was no significant difference between the two. Fibrinogen levels were variably reduced to a minor degree.

Plasma prothrombin times (Quick and Ware-Stragnell) were prolonged to an insignificant degree by both substances. True prothrombin assays showed no change. Serum prothrombin times (Figure 5) showed a profound effect. Prothrombin consumption was completely in-

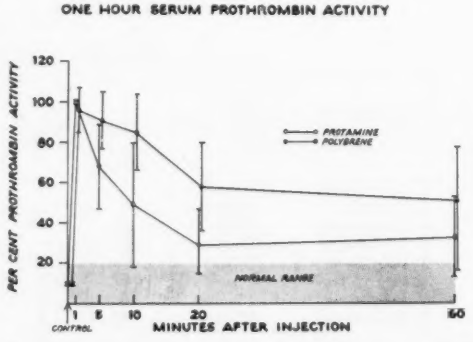

FIG. 5. ONE-HOUR SERUM PROTHROMBIN ACTIVITY. Per cent prothrombin activity in serum 1 hour after clotting as related to activity of plasma from same blood sample.

hibited immediately after injection, and was still abnormal 60 minutes later, long after the platelet count had returned above levels at which it could be responsible for the results. Although the difference was not statistically significant, as shown by the standard deviations in the figure, the effect of Polybrene appeared to be greater than that of protamine.

Cephalin times (Figure 6) also showed a marked abnormality, and were not back to the control levels by the end of an hour. Again Polybrene showed the greatest effect although the differences do not appear to be statistically significant. The changes in clotting time, prothrombin consumption, and cephalin times were greater and more persistent than could possibly be explained by the anticoagulant effect of circulating protamine or Polybrene. Proof that the abnormality was due to a clotting factor deficiency rather than to a circulating anticoagulant is provided in Figure 7, which shows the cephalin times obtained when control plasma was mixed in varying proportions with samples obtained 2, 10 and 60 minutes after Polybrene. In the 2-minute sample, when the Polybrene level was at its highest, a slight anticoagulant effect is apparent in that 10 per cent of the 2-minute plasma prolonged the cephalin time of the control plasma, but even with the 2-minute sample, 10 per cent of control plasma had a significant shortening effect. The ability of a small amount of normal plasma to correct the abnormal plasma (characteristic of a clotting factor deficiency) is clearly apparent with the 10- and 60-minute samples.

The abnormal results with whole blood clotting times, cephalin times, and prothrombin consumptions (compared with the normal prothrombin

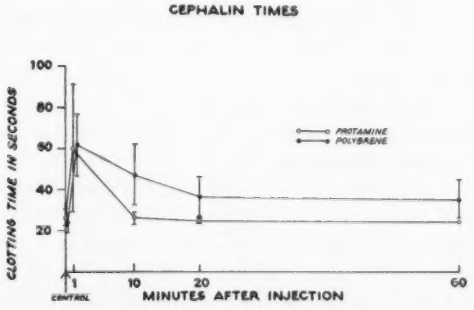

FIG. 6. CEPHALIN TIMES.

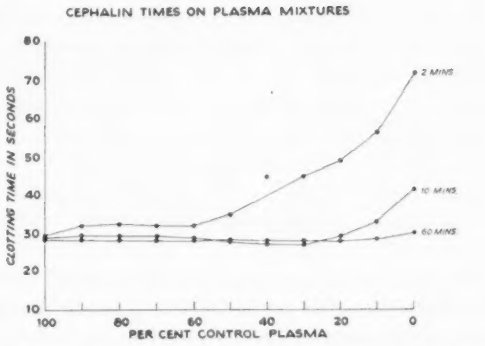

FIG. 7. CEPHALIN TIMES ON PLASMA MIXTURES. Control plasma was mixed in varying proportions with plasma from a sample removed 2 minutes after the injection of Polybrene, and cephalin times of the mixtures were determined. Mixtures of control plasma with plasma obtained 10 and 60 minutes after Polybrene were similarly checked.

times) indicated that the clotting factor deficiency was in the first stage of coagulation, the stage of thromboplastin generation. Despite this, thromboplastin screening and generation tests by the standard techniques showed little or no abnormality. Evidence that thromboplastin generation was impaired, however, was obtained using highly diluted reagents.

Dog platelets tested in the thromboplastin generation test (TGT) at intervals after injection of

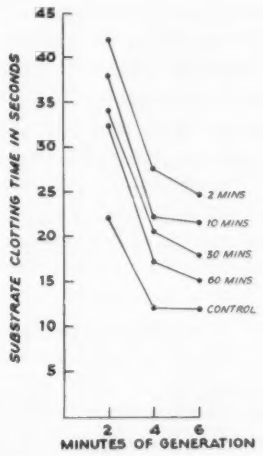

FIG. 8. THROMBOPLASTIN GENERATION TEST (CONTROL SERUM AND CEPHALIN). The effects of absorbed plasma obtained before and at various intervals after Polybrene are compared in the TGT.

TABLE III

Factor V levels (per cent control) after injection of Polybrene (5 mg/kg)

Method	No. of expts.	Control	Minutes after injection 2	5	10	20	60
Ref. 11	7	100	171 ± 47*	173 ± 50	163 ± 46	141 ± 39	90 ± 37
Ref. 12	1	100	200	168	100		

* Standard deviation.

Polybrene remained qualitatively normal, even when employed as washed whole platelets at the suboptimal concentration of 100,000 per mm^3.

Serum is customarily employed in the TGT on human blood at a dilution of 1:10. Using control dog serum it was demonstrated that, in this animal, serum could be diluted as high as 1:40 and still produce a potent thromboplastin in 6 minutes. The post-Polybrene samples generated thromboplastin normally at this dilution.

Citrated plasma absorbed with aluminum hydroxide is used to provide Factor VIII and Factor V for thromboplastin generation. The standard test employs a dilution of 1:5. At optimal dilution for the dog (1:40 also) the absorbed plasma from blood samples taken after Polybrene showed a marked defect, which gradually returned toward normal, but was still far from the control levels at the end of 1 hour (Figure 8).

It was unlikely that the defect in absorbed plasma was due to a Factor V deficiency, since this would have prolonged the prothrombin times. Specific assays for Factor V showed the rather unexpected result that Factor V levels were actually increased by Polybrene (Table III).

By exclusion we were thus left with a Factor VIII deficiency as the explanation of our abnormal clotting tests. Direct assays for Factor VIII by a very sensitive technique proved that this was indeed true, and the specificity of the results was corroborated by an assay using plasma from a patient with classical hemophilia as the substrate. The severe depression of antihemophilic globulin levels and the prolonged duration of the defect are very striking (Table IV, line 1). Control levels of Factor VIII in the dogs used were more than five times as high as the average of our normal humans. This explains why the antihemophilic globulin defect became apparent in the TGT only in systems using highly diluted plasmas.

A slower infusion of the same dose of Polybrene (Table IV, line 2) resulted in a slower, milder depression of Factor VIII levels with return toward normal beginning by the end of 1 hour. A second rapid injection given 1 hour after the first (Table IV, line 3), resulted in no further fall in Factor VIII levels despite the fact that simultaneous assays of Polybrene levels showed them to be much higher than those after the first injection (Table II, line 8). When heparin (6 mg per kg) was injected prior to the Polybrene (two experiments) there was no change in Factor VIII from the control value. When heparin was injected 10 minutes after Polybrene (two experiments) it had no effect on the course of the low Factor VIII levels.

DISCUSSION

The demonstration that a synthetic basic compound of large molecular weight (Polybrene)

TABLE IV

Factor VIII levels (per cent control) after injection of Polybrene (5 mg/kg)

Rate of injection of Polybrene	No. of expts.	Control	Minutes after injection 2	5	10	20	60
1 Fast	7	100	22 ± 28	8 ± 8	7 ± 5	10 ± 8	8 ± 2
2 Slow*	6	100	82 ± 23	50 ± 27	26 ± 30	29 ± 30	60 ± 32
3 Fast†	4	14	13	12	12	11	

* The undiluted Polybrene was infused by a slow drip during the first 10 minutes of sampling.
† Given 60 minutes after a fast injection of Polybrene.

could be used to neutralize heparin *in vivo* (15) has led to a number of studies to compare its effects with those of the earlier used heparin antagonist, protamine (16–18). In two of these studies, it was concluded that Polybrene is to be preferred because, following its use, less postoperative blood loss was noted. These studies discounted (perhaps erroneously) the possibility that the lesser degree of postoperative bleeding might be due to improved techniques of patient management and did not consider the possibility that the dose of Polybrene selected may have come closer to exact neutralization of heparin than did the dose of protamine previously used.

One criticism of protamine (16) was that it was unstable and varied in potency. The references quoted in support of this idea were to preparations of protamine available a number of years ago, and this objection is no longer valid according to our experience.

Since early work with Polybrene demonstrated that it could produce the same side effects as protamine (15), we performed a series of experiments on dogs in which the magnitude of the effects of the two heparin antagonists could be compared with some exactness. Because protamine clearly had been shown to have an anticoagulant effect *in vitro* (19, 20), we were particularly interested in finding out whether this was demonstrable after *in vivo* injection and whether the findings would be similar with Polybrene.

The results reported above show that there is a marked variability in the responses of individual dogs. Nonetheless, it is apparent that in comparable doses protamine and Polybrene show the same tendency to produce marked hypotension, tachycardia followed by bradycardia, and slowing of respiration followed by tachypnea. Similarly, both drugs demonstrated an equal tendency to induce thrombocytopenia and an unexpected defect of the plasma coagulation system. Although studies demonstrated that Polybrene was a more powerful heparin-neutralizing agent than protamine, it also had a slightly more depressing effect on the blood clotting system.

Polybrene was demonstrated to disappear from the circulating blood very rapidly, as previously had been shown for protamine (21). The mechanism by which they disappear is unknown but their site of uptake appears to have a limited capacity in view of the much higher blood levels achieved when a second injection followed the first. The coagulation defect induced by both drugs persisted long after the heparin antagonists had left the blood stream, and could not be entirely explained by their anticoagulant effect nor by the induced thrombocytopenia. The prolonged whole blood clotting and cephalin times and the poor prothrombin consumption, as compared with normal plasma prothrombin times, suggested that a defect had been induced in the first stage of blood coagulation. Although standard thromboplastin generation and screening tests failed to establish this, more sensitive tests clearly demonstrated that a striking deficiency of Factor VIII had been induced. The animals bled persistently at the site of vessel cannulation, and it thus appears that a temporary coagulation defect had been induced analogous to classical hemophilia.

The mechanism by which Factor VIII is removed from the circulating blood is unclear. The magnitude and duration of the decrease in Factor VIII levels is related to the dose of Polybrene given and the speed at which it is injected. This suggests that the depression of Factor VIII depends on the blood level of Polybrene achieved and that the two may form a complex which is then removed from the circulation. Evidence against this supposition, however, is provided by the fact that a second injection of Polybrene, given while Factor VIII was still at a very low level, did not succeed in reducing the concentration of the coagulation factor further, even though much higher levels of Polybrene appeared in the circulating blood than those following the first dose.

Further objection to the hypothesis of direct interaction between Polybrene and Factor VIII comes from the fact that such a reaction cannot be demonstrated in the test tube. Protamine inhibits thromboplastin generation *in vitro* but it has not been possible to demonstrate that it acts by opposing Factor VIII. In fact, Shanberge (20) believes it may react with Factor IX. His evidence for this was that serum containing Factor IX appeared to promote thromboplastin generation in the presence of protamine. Shanberge's evidence does not prove direct interaction between protamine and Factor IX; moreover, Hougie (19) was unable to confirm Shanberge's claim that serum had a specific antagonistic action against

protamine in the TGT. In any case, no evidence can be offered to show that protamine and Factor VIII react with each other *in vitro*. One could speculate that a protamine- or Polybrene-Factor VIII complex does form, that *in vivo* it is cleared from the circulation, but that *in vitro* it remains available for products of beginning coagulation to attract Factor VIII and thus disrupt the complex The best argument against the direct interaction between Polybrene and Factor VIII remains the failure of a second dose of Polybrene to lower the Factor VIII levels further.

A second possible explanation for the disappearance of Factor VIII is that it is consumed by beginning intravascular coagulation. This possibility gains weight when we consider the fact that the whole blood clotting times momentarily shortened before they became prolonged, that prior injection of heparin prevented the loss of Factor VIII and that the platelet count fell. On the other hand, intravascular coagulation should consume Factor V, prothrombin and fibrinogen, but these did not decrease. Moreover, thrombocytopenia occurs even with prior heparin. One might speculate that the process of intravascular coagulation was interrupted by the anticoagulant action of Polybrene at some point between the consumption of Factor VIII and that of the other factors mentioned or that just enough thrombin evolved to destroy Factor VIII alone. No evidence can be offered that this is possible.

It seems most likely at the moment that Factor VIII disappears, not by means of direct combination with Polybrene and not as a result of beginning intravascular coagulation, but in a more indirect manner. Failure of a second injection of Polybrene to lower the Factor VIII level further suggests that some compound intermediary in the reaction may have been depleted by the first injection.

The changes in Factor V following injection of Polybrene were in contrast to those in Factor VIII. The levels rose sharply to nearly twice the control values and then returned to normal over a period of 60 minutes. No obvious explanation for these changes is apparent. When Polybrene is added to plasma *in vitro*, apparent levels of Factor V decrease as the quantity of Polybrene is raised. The rise in Factor V appears to be a true one, since one of the assays used is believed to fully activate Factor V before it is measured (11).

The findings presented here have definite practical implications. Andersen, Mendelow and Alfano have observed (22) that clotting times of whole blood after heart-lung bypass and neutralization of heparin are frequently longer than the control preoperative values. They believe that greater abnormalities occur with larger doses of heparin antagonists. These findings suggest that our observations in dogs are probably a reflection of what occurs in human patients. The clotting defect induced will vary in degree according to the amount of Polybrene or protamine that is administered in excess of that which is neutralized by the available heparin. In the experiments reported here, a dose of Polybrene of 5 mg per kg was administered to ensure that the clotting defect would be sufficiently severe for analysis. Most surgical teams give Polybrene in a dose approximately equal to that which has been used for heparin. It is highly unlikely under these conditions that an excess of Polybrene as high as 1 mg per kg would be given. Such an excess would produce a mild and transient clotting defect of little clinical significance. Larger doses, however, would carry a real risk; and the chief danger is that the surgeon, confronted by a patient with a prolonged clotting time and excessive bleeding, will give additional doses of Polybrene or protamine under the assumption that heparin has not yet been completely neutralized. Moreover, there are still recommendations appearing in the current literature that doses of protamine or Polybrene can or should be four to eight times the dose of heparin which had been administered (23). Most surgical teams have arrived empirically at a dose of heparin antagonist which works for them. It is our belief that this dose should not be exceeded unless it can be demonstrated in the test tube that added protamine or Polybrene will shorten the clotting time of the patient's blood.[3]

[3] Dr. John Osborn at the Presbyterian Hospital in San Francisco suggests a very simple way to do this. He dilutes Polybrene 1:40 with saline in a syringe, then transfers 1 drop to an empty 12 × 75 mm glass tube, 2 drops to a second, 3 drops to a third, and leaves a fourth tube empty. One ml blood is added to each tube, mixed with the Polybrene and observed for clotting at room

We hasten to add that we do believe it important that all traces of unneutralized heparin be eliminated in these cases (23).

Since protamine or Polybrene in appropriate doses can neutralize heparin equally well, and since they demonstrate the same undesirable side effects to approximately the same degree, there is little reason to prefer one to the other. It would seem, therefore, that the choice of drug is less important than the choice of dosage, particularly in regard to avoiding the production of a paradoxical prolongation of clotting by either agent.

SUMMARY

Two heparin antagonists, protamine and Polybrene, have been compared in a series of parallel experiments *in vivo* and *in vitro*. The effects of both drugs on blood pressure, pulse, respiration, platelet counts, and blood coagulation are so similar that there is little reason to prefer one over the other. Polybrene neutralizes more heparin than a comparable dose of protamine, but the degree of undesirable side effects appears to correlate with heparin-neutralizing ability.

Large doses of either drug have the unexpected capacity of inducing a striking deficiency of Factor VIII in dogs. Factor V levels are, in contrast, elevated. The mechanism by which these effects are produced is not clear, but it seems probable that the reaction is a nonspecific one and unrelated to the well known anticoagulant effects of these heparin antagonists. It is likely that a similar antihemophilic globulin deficiency may be induced in human patients by the use of excessive doses of heparin antagonists following open heart surgery. The dangers of this situation are obvious, and a plea is made for judicious use of the heparin antagonists.

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temperature every few minutes. This is a simplification of a method we have previously reported (24).

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STUDIES OF RESPIRATORY PHYSIOLOGY IN CHILDREN. VI. LUNG DIFFUSING CAPACITY, DIFFUSING CAPACITY OF THE PULMONARY MEMBRANE AND PULMONARY CAPILLARY BLOOD VOLUME IN CONGENITAL HEART DISEASE *

By GIOVANNI BUCCI and CHARLES D. COOK with the technical assistance of JOANNA F. HAMANN

(From the Department of Pediatrics, Harvard Medical School, and the Department of Medicine of the Children's Hospital Medical Center, Boston, Mass.)

(Submitted for publication January 12, 1961; accepted April 13, 1961)

Studies of lung diffusing capacity for carbon monoxide (D_{LCO}) at various alveolar O_2 tensions (1, 2) and on the kinetics of the carboxyhemoglobin reaction (3, 4) have made possible the calculation of the diffusing capacity of the pulmonary membrane (D_M) and the pulmonary capillary blood volume (V_c) (5). In the present study D_{LCO}, D_M and V_c have been determined in 34 patients with various types of congenital heart disease, compared to previous studies in normal controls (6) and related to hemodynamic data derived from cardiac catheterizations. The effect of exercise on the pulmonary capillary bed of one patient with a left-to-right shunt has been studied, and in ten patients carbon monoxide diffusion studies have been repeated after surgical correction of the cardiac malformation.

SUBJECTS AND METHODS [1]

Although the patients ranged in age from 8 to 42 years, most were children, only 5 of them being over 18 years. None of the patients had any known primary pulmonary abnormalities and none had clinical signs of congestive heart failure. Cardiac diagnoses were established by the usual criteria including cardiac catheterization (7) [2] (in all but Patient 15). The diagnosis was confirmed at operation in 28 of the 34 cases including Patients 15 and 21. In Patient 20 the diagnosis of a hypoplastic left heart was established at autopsy. Only pulmonary blood flows and mean pulmonary artery wedge pressures obtained within 20 days of the diffusion study were used for examining the relations of these factors to D_M and V_c.

The pertinent clinical and laboratory data for the individual patients are given in Tables I and II. The patients are grouped partly according to diagnosis and partly according to their hemodynamic status. In group I are 24 patients with increased pulmonary blood flow ($\dot{Q}_P$). Of the 21 patients with atrial septal defect (ASD), the ostium secundum type was an isolated lesion in 13, associated with partial anomalous pulmonary venous return in 3 and with mild pulmonic stenosis in 1. Two patients had an ostium primum septal defect and associated significant mitral regurgitation. In Patients 20 and 21, conditions (a hypoplastic left heart and pericarditis, respectively) apt to produce increased pulmonary venous pressure were associated with the ASD. Three patients with ventricular septal defect (VSD) are included in this group with increased $\dot{Q}_P$.

Six patients with normal or decreased $\dot{Q}_P$ make up group II. Four of these had pulmonic stenosis (PS), one had tetralogy of Fallot (TOF) and one had tricuspid atresia. In group III are four cases with congenital or acquired aorto-pulmonary shunts and other associated conditions. These patients are separated because their $\dot{Q}_P$ status had varied with surgery or acquired pulmonary vascular changes.

Lung volumes were determined in duplicate by means of a spirometer and a closed circuit helium dilution method (8). D_{LCO}'s at high and low O_2 concentrations (90 and 21 per cent, respectively, for the inspired gas mixture) were obtained from the 10-second breath-holding technique described by Forster and co-workers (9, 10) and stand-

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[1] Abbreviations used in this paper are: D_{LCO} = total lung diffusing capacity for carbon monoxide (ml/mm Hg/min); D_M = diffusing capacity of the pulmonary membrane (ml/mm Hg/min); V_c = pulmonary capillary blood volume (ml); R_M/R_c = the ratio of the membrane to intracapillary resistance to diffusion; $\dot{Q}_P$ = pulmonary blood flow (L/min/m² BSA); $\bar{P}_{CO_2}$ = mean capillary oxygen tension (mm Hg); TLC = total lung capacity (L); ASD = atrial septal defect; VSD = ventricular septal defect; PS = pulmonic stenosis; TOF = tetralogy of Fallot.

[2] Cardiac catheterizations were performed in the cardiopulmonary laboratory of the Children's Hospital Medical Center; we are indebted to Drs. A. S. Nadas and A. M. Rudolph for the opportunity to study the patients and permission to use the catheterization data.

TABLE I

Clinical and pulmonary function data in patients with congenital heart disease

Patient no.	Diagnosis	Sex	Age	Ht	Wt	BSA	TLC	D_{LCO}	D_M	V_c
			yrs	*cm*	*kg*	m^2	*L*	*ml/mm Hg/min*		*ml*
			Group I							
1	ASD	M	9	128	24	0.91	2.26	19.3	37	61
2	ASD	F	9	125	21	0.85	2.05	15.3	30	47
3	ASD	F	10	132	25	0.96	2.36	21.3	42	71
4	ASD	F	12	150	38	1.27	2.84	22.0	49	61
5	ASD	M	14	156	48	1.40	3.94	26.7	64	60
6	ASD	M	14	164	55	1.58	4.53	27.5	55	83
7	ASD	F	15	147	38	1.25	2.28	23.0	51	65
8	ASD	M	16	168	44	1.46	4.12	41.5	81	123
9	ASD	F	16	165	55	1.58	4.65	32.1	62	97
10	ASD	M	17	175	53	1.63	5.93	41.8	96	112
11	ASD	F	18	163	57	1.58	4.37	32.0	63	110
12	ASD	M	26	178	79	1.98	6.89	47.7	86	151
13	ASD	F	35	163	49	1.46	4.30	27.6	77	83
14	ASD } partial anomalous pulmonary venous return	F	8	130	24	0.93	2.51	19.5	43	49
15	ASD } partial anomalous pulmonary venous return	M	10	136	28	1.02	2.89	20.1	48	55
16	ASD } partial anomalous pulmonary venous return	F	18	154	63	1.50	4.36	32.0	70	89
17	ASD, mild pulmonic stenosis	M	8	129	25	0.93	2.22	23.0	46	74
18	ASD, mitral regurg.	M	12	150	46	1.40	3.30	22.9	35	129
19	ASD, mitral and tricuspid regurg.	M	15	170	59	1.68	4.90	43.0	94	119
20	ASD, hypoplastic left heart	F	16	151	40	1.31	2.49	25.4	45	98
21	ASD, pericarditis	M	42	173	76	1.90	4.70	32.4	54	114
22	VSD	M	11	145	32	1.14	3.17	23.3	48	73
23	VSD, mild pulmonic stenosis	F	11	130	22	0.90	1.84	12.6	26	38
24	VSD, pulmonary vascular obstruction	M	9	139	23	0.96	2.31	18.3	30	69
			Group II							
25	Pulmonic stenosis, moderate	M	9	122	22	0.86	2.24	15.2	35	37
26	Pulmonic stenosis, severe	M	11	142	28	1.06	2.95	13.1	26	34
27	Pulmonic stenosis, severe	M	11	134	25	0.96	2.71	14.8	34	35
28	Pulmonic stenosis, moderate	M	12	152	41	1.32	3.31	20.7	47	52
29	Tetral. of Fallot	M	11	128	24	0.96	2.50	10.0	19	28
30	Tricuspid atresia	M	19	175	55	1.65	4.97	42.7	81	82
			Group III							
31	Tetral. of Fallot, Pott's proced. 4 yrs previously	F	12	129	21	0.94	1.99	15.6	29	50
32	Tetral. of Fallot, Pott's proced. 6 yrs previously	M	15	155	68	1.44	3.84	24.4	48	73
33	Tetral. of Fallot, Pott's proced. 6 yrs previously; pulmonary vascular obstruction	M	33	164	56	1.61	3.66	23.0	41	58
34	Patent ductus arteriosus, pulmonary vascular obstruction	M	17	171	52	1.59	5.53	27.2	63	60

ardized by Ogilvie, Forster, Blakemore and Morton (11). A small but significant and progressive decrease in D_{LCO} during successive determinations at both high and low O_2 tensions was found in the patients with congenital heart disease. Carbon monoxide accumulation could not have been enough to account for this (9). Corresponding variations in alveolar volume, breath-holding time or possible systematic errors in gas analysis have not been detected, and since a very similar pattern was observed in the normal controls (6), no correction was made for this variation. The calculations of D_M, V_c [3] and the ratio of membrane to intracapillary re-

[3] These relations are shown by the equation:

$$1/D_{LCO} = 1/D_M + 1/\theta V_c$$

where θ is a constant related to the rate of uptake of CO by hemoglobin at various O_2 concentrations (3–5).

TABLE II

Pulmonary function studies and cardiac catherization data in patients with congenital heart disease

Patient no.	Diagnosis	Pulmonary function studies as % of predicted values				Heart catheterization studies					
		TLC*	D_{LCO}*	D_M*	V_c*	R_M/R_c†	Time‡	$\dot{Q}_P$§	Pulmonary wedge pressure	Pulmonary‖ vascular resistance	Hb
								L./min/m²	*mm Hg*	*mm Hg/L./min/m²*	*g%*
					Group I						
1	ASD	95	136	132	160	1.11	20 d	10	5	0.5	13.0
2	ASD	96	114	115	131	1.13	4 y	13	9.5	0.3	13.8
3	ASD	91	146	145	187	1.19	5 y	11	7		12.3
4	ASD	76	134	144	142	0.89	3 y	12	7.5		13.6
5	ASD	93	129	139	113	0.67	1 d	10	5	0.8	16.3
6	ASD	95	120	104	141	1.08	1 d	10	12	0.6	13.6
7	ASD	65	161	182	171	0.91	4 y	12	4		13.6
8	ASD	81	194	169	224	1.06	4 m	16	10	0.3	14.9
9	ASD	97	137	115	161	1.13	6 m	12	6	0.3	14.4
10	ASD	104	183	141	156	0.83	3 y	7	9	0.5	14.0
11	ASD	94	144	120	193	1.20	1 d	8	5	1.0	13.8
12	ASD	113	149	109	186	1.20	2 m	12			14.8
13	ASD	91	125	154	148	0.76	3.5 m	14	9	0.5	10.6
14	ASD } partial anomalous pulmonary venous return	101	128	139	122	0.81	1 d	9	2	1.0	14.6
15	ASD } partial anomalous pulmonary venous return	102	121	137	128	0.81					13.0
16	ASD } partial anomalous pulmonary venous return	109	144	137	156	0.92	7 m	8		1.1	13.5
17	ASD, mild pulmonic stenosis	91	164	170	200	1.15	3 y	17	5	0.6	12.7
18	ASD, mitral regurg.	89	126	90	274	2.63	1 d	10	11	0.7	11.1
19	ASD, mitral & tricuspid regurg.	93	177	165	192	0.92	16 d	8	10	0.7	14.4
20	ASD, hypoplastic left heart	66	168	150	245	1.58	4 y				11.8
21	ASD, pericarditis	84	138	98	190	1.54	4 d	7	15	2.2	13.6
22	VSD	94	131	126	159	1.08	1 d	11	6	0.6	13.4
23	VSD, mild pulmonic stenosis	74	100	113	112	1.02	1 d	21	9	1.1	12.4
24	VSD, pulmonary vascular obstruction	79	127	136	182	1.62	1 d	12	16	3.9	13.6
	Average	92	142	134	170	1.14		11	8.2	0.9	14.0
	Significance of difference of average from normal p =		<0.001¶	<0.001¶	<0.001¶	<0.001¶					
					Group II						
25	Pulmonic stenosis, moderate	108	108	125	100	0.73	1 y	4.7	4	0.6	15.7
26	Pulmonic stenosis, severe	93	78	75	77	0.94	1 d	2.2	5	2.2	16.0
27	Pulmonic stenosis, severe	100	93	103	83	0.74	1 d	2.2	7	2.0	15.0
28	Pulmonic stenosis, moderate	86	113	120	110	0.78	1 d	3.6	4	2.0	14.3
29	Tetral. of Fallot	105	66	63	70	1.02	1 d	2.4	2.5	2.4	15.6
30	Tricuspid atresia	87	173	140	130	0.72	1 d	3.2			24.0
					Group III						
31	Tetral. of Fallot, Pott's proced. 4 yrs previously	82	118	116	143	1.30	1 d	9.4	9	1.7	13.7
32	Tetral. of Fallot, Pott's proced. 6 yrs previously	95	120	104	140	1.07	1 d	4.2	7	1.7	14.0
33	Tetral. of Fallot, Pott's proced. 6 yrs previously; pulmonary vascular obstruction	76	117	107	141	1.01	1 d	3.5	8	17.0	18.8
34	Patent ductus arteriosus, pulmonary vascular obstruction	103	101	98	88	0.67	2 y	4.2		15.4	17.0

* TLC predicted from height; other values predicted from actual TLC.
† Membrane/intracapillary resistance ratio.
‡ Time = interval between catheterization and pulmonary studies in days (d), months (m) or years (y).
§ Pulmonary blood flow in L/min/m² BSA; normal = 3 to 5 (7).
‖ Normal = 3 mm Hg/L/min/m² or less.
¶ Standard deviations of D_{LCO}, D_M, V_c and R_M/R_c in normal subjects are ±12.5, 20.5, 15.0, and ±0.17%, respectively. (Student's *t* test used.)

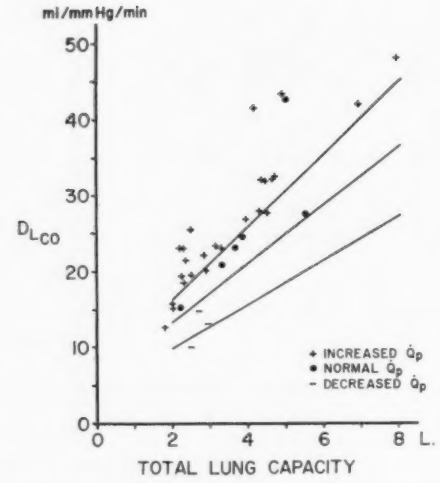

FIG. 1. LUNG DIFFUSION (D_{LCO}) VS TOTAL LUNG CAPACITY IN 34 PATIENTS WITH CONGENITAL HEART DISEASE. The three lines represent the average and 2 standard deviations around the average for normal subjects (6). Pulmonary blood flow ($\dot{Q}_P$) was considered increased if more than 5 L/min/m² and decreased if less than 3 L/min/m². Twenty of the 25 patients with increased $\dot{Q}_P$ have a significantly elevated D_{LCO}.

sistance to diffusion (R_M/R_c) [4] followed the methods and equations of Roughton and Forster (5) and of McNeill, Rankin and Forster (12). More details of the corrections and calculations employed are given in a previous paper (6).

Since it has been previously shown that diffusion studies in normal children and adults correlate well with TLC (6), this parameter was used as a basis for prediction of the expected values for the patients. The individual determinations have been expressed as per cent of the predicted values. Similar comparisons were made using surface area as a basis for prediction; the changes were slightly smaller, but in the same direction and are not reported. TLC values were compared on the basis of height to our normal standards except that normal values for adult men were derived from the data of Hepper, Fowler and Helmholz (13).

In the cardiac patients, the SE of D_{LCO} determined in triplicate was ± 1 per cent. Repeated determinations in one normal subject of D_M and V_c, when based on three D_{LCO} determinations at both high and low O_2 concentrations, had SD's of ± 17 and ± 8 per cent, respectively. In one previously reported adult patient (6) with atrial septal defect and an elevated total lung diffusing capacity, similar variations in the D_M and V_c values were found.

[4] $R_M/R_c = D_{LCO}/D_M - D_{LCO} \times 14.9/Hb\,(g\%)$ where D_{LCO} is the estimated diffusing capacity at 120 mm Hg P_{CO_2}.

For the predicted values of D_{LCO}, D_M and V_c, 2 SD's around the mean values averaged ± 25, 41 and 30 per cent, respectively. Two SD's around the mean predicted value for TLC averaged ± 27 per cent.

For the exercise studies a bicycle ergometer [5] was used; the subjects were in the sitting position for this and all pulmonary tests. Since a steady circulatory state (14) and a steady D_{LCO} (15) are apparently reached after 2 minutes of exercise, all measurements of diffusion were performed at the beginning of the third minute of cycling. A moderate work load corresponding to a cardiac rate of 139 to 153 beats per minute was selected (16). The alveolar CO_2 and O_2 concentrations at the beginning and end of breath-holding and the O_2 consumption during exercise were also determined and used for the calculation of D_{LCO} at 120 mm Hg P_{CO_2} and for the corrected $1/\theta$ values.

RESULTS

The results of the studies are shown in Tables I and II. As indicated in Figures 1, 2 and 3, where individual values for D_{LCO}, D_M and V_c are plotted against TLC and compared with the normal ranges previously reported (6), these values average significantly ($p < 0.001$) above normal in patients with an elevated $\dot{Q}_P$ and in the normal

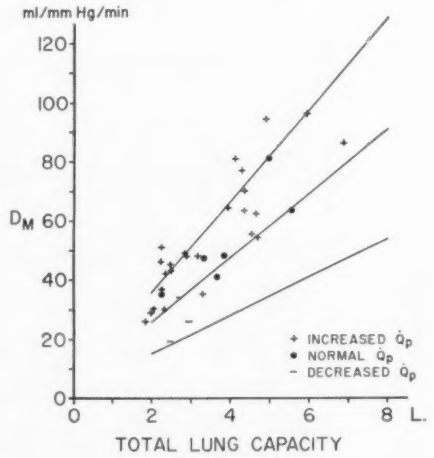

FIG. 2. MEMBRANE DIFFUSION (D_M) VS TOTAL LUNG CAPACITY IN 34 PATIENTS WITH CONGENITAL HEART DISEASE. The three lines represent the average and 2 standard deviations around the average for normal subjects (6). Pulmonary blood flow ($\dot{Q}_P$) was considered increased if more than 5 L/min/m² and decreased if less than 3 L/min/m². Eight of the 25 patients with increased $\dot{Q}_P$ have a significantly elevated D_M.

[5] Elema Ergometer, Stockholm, Sweden.

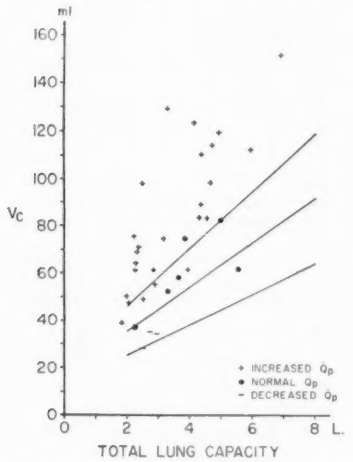

FIG. 3. PULMONARY CAPILLARY BLOOD VOLUME (V_c) VS TOTAL LUNG CAPACITY IN 34 PATIENTS WITH CONGENITAL HEART DISEASE. The three lines represent the average and 2 standard deviations around the average for normal subjects (6). Pulmonary blood flow ($\dot{Q}_P$) was considered increased if more than 5 L/min/m² and decreased if less than 3 L/min/m². Twenty-one of the 25 patients with increased $\dot{Q}_P$ have a significantly elevated V_c.

range in patients with normal [6] or low $\dot{Q}_P$. In addition, irrespective of $\dot{Q}_P$, all patients in whom a tetralogy of Fallot malformation had been treated with a Pott's anastomosis had an elevated V_c; Patient 34 with a patent ductus arteriosus and pulmonary vascular obstruction of long duration had low normal values. Within the group of patients with increased Q_P no correlation between the degree of increase and the V_c could be found.

If the patients with ASD are grouped according to the presence of conditions leading to increased pulmonary venous pressure (Table III), it is found that V_c is significantly ($p < 0.005$) elevated in those with such complicating lesions (on the average 225 per cent of predicted, as compared with 160 per cent in the uncomplicated group). In the small number of these patients with increased Q_P in whom a comparison could be made on the basis of wedge pressure under or over 10 mm Hg, the V_c averaged more in those with an elevated pressure, although this difference was not

[6] In one patient with a normal $\dot{Q}_P$ (Patient 30), D_{LCO} was > 2 SD's above the predicted value; at least part of this increase was on the basis of a markedly elevated hemoglobin concentration.

TABLE III

Comparison of groups of patients with increased pulmonary blood flow

	Patients	No. of cases	TLC*	D_{LCO}*	D_M*	V_c*	Catheterization data		
							R_M/R_c	$\dot{Q}_P$	Pulmonary wedge pressure
								L/min/m²	*mm Hg*
ASD, without conditions tending to produce increased pulmonary venous pressure	1–17	17	94	143	138	160†	0.99†	11	7
ASD, with conditions tending to produce increased pulmonary venous pressure	18–21	4	83	152	126	225†	1.67†	8	12
Increased $\dot{Q}_P$ $\bar{P}_c \leq 10$ mm Hg	1, 5, 11, 14, 19, 22, 23‡	7	92	135	133	150	0.97§	11	6
Increased $\dot{Q}_P$ $\bar{P}_c > 10$ mm Hg	6, 18, 21, 24‡	4	88	129	107	197	1.72§	10	13

* TLC as per cent of predicted on the basis of height; D_{LCO}, D_M, V_c as per cent of predicted on the basis of actual TLC.

† Significant difference ($p < 0.005$) between the averages for Patients 1–17 and for Patients 8–21 using Student's *t* test.

‡ Only patients whose cardiac catheterization was performed within 20 days of the pulmonary studies have been included in these two groups.

§ Significant difference ($p < 0.025$) between the averages for cases with low and with high wedge pressures.

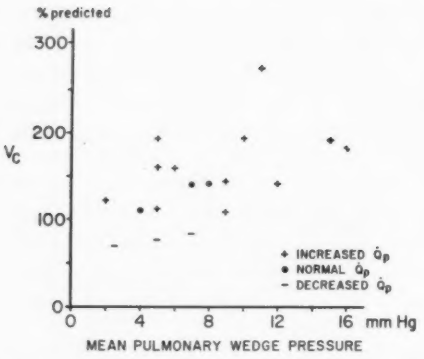

FIG. 4. PULMONARY CAPILLARY BLOOD VOLUME (V_C) VS MEAN PULMONARY ARTERY WEDGE PRESSURE IN THE 18 PATIENTS WITH CONGENITAL HEART DISEASE WHO WERE CATHETERIZED WITHIN 20 DAYS OF THE DIFFUSION STUDIES. V_c's are expressed as per cent of the value predicted on the basis of the patient's actual total lung capacity (6). Pulmonary blood flow ($\dot{Q}_P$) was considered increased if more than 5 L/min/m² and decreased if less than 3 L/min/m². The significant ($p < 0.05$) trend toward increasing V_c with increasing wedge pressure is apparent. (Chi square test was used to compare patients with wedge pressures above and below 10 mm Hg.)

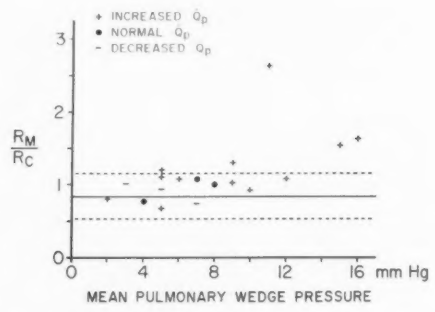

FIG. 5. THE RATIO OF THE MEMBRANE TO INTRACAPILLARY RESISTANCE TO DIFFUSION (R_M/R_C) VS MEAN PULMONARY WEDGE PRESSURE. The three lines represent the average and approximately 2 standard deviations around the average for normal subjects (6). Pulmonary blood flow ($\dot{Q}_P$) was considered increased if more than 5 L/min/m² and decreased if less than 3 L/min/m². The trend toward increasing membrane resistance relative to the intracapillary resistance with increasing wedge pressure is shown.

significant. When the V_c's are plotted (Figure 4) against wedge pressures for all patients catheterized within 20 days of the pulmonary studies, a significant ($p < 0.05$) trend toward increasing V_c with increasing wedge pressure is apparent. It is also shown in Figure 4 that even when the wedge pressures were similar, patients with low pulmonary blood flow showed smaller V_c's.

In group I, D_M is not increased as much as V_c, and hence R_M/R_c is on the average significantly ($p < 0.001$) greater than in normal subjects (1.14 ± 0.39 vs 0.81 ± 0.17) (6). The highest values of R_M/R_c were found in patients with the highest wedge pressures (Table III and Figure 5). In group II the R_M/R_c ratio is normal (0.82).

Patient 12 with an ASD was studied during exercise to see if this procedure might further increase the pulmonary capillary blood volume. On moderate exercise this patient's D_{LCO} increased from 149 per cent of predicted to 218 per cent, D_M from 109 to 165 per cent, and V_c from 186 to 245 per cent. These increases with exercise, when expressed as percentage of the control values, were similar to those found for two normal adults who were subjected to approximately the same exercise load (Table IV).

In ten patients diffusion studies were repeated at least 3 months after surgical correction of their cardiac malformation (Table V). In the eight patients with ASD or VSD, D_{LCO}, D_M and V_c decreased on the average to within normal limits

TABLE IV

Effect of exercise on diffusion studies *

Subject	Age	Diagnosis	No. of determin. Rest	No. of determin. Exercise	Exercise	Heart rate	D_{LCO}†	D_M	V_c	R_M/R_c
	yrs				*kg-m/min*					
GB	32	Normal	1	3	840	+113	+28	+8	+45	+40
DC	41	Normal	3	2	1,000	+107	+21	+14	+28	+12
12	26	ASD	4	1	870	+ 87	+46	+51	+32	− 6

* Change during exercise expressed as per cent variation from resting values.

† D_{LCO} calculated for 120 mm $\bar{P}_{CO_2}$.

TABLE V

Pre- and postoperative studies

Patient no.	Diagnosis	Time of study	TLC	D_{LCO}	D_M*	V_c*	R_M/R_c
			L				
			Group I: increased $\dot{Q}_p$				
2	ASD	Pre-op.	2.05	114	115	131	1.13
		10 mos postop.	2.16	78	81	96	1.13
		Difference		−36	−34	−35	0
3	ASD	Pre-op.	2.36	146	145	187	1.19
		4 mos postop.	2.48	97	96	110	1.06
				−49	−49	−77	−0.13
6	ASD	Pre-op.	4.53	120	104	141	1.08
		4 mos postop.	4.69	102	84	134	1.18
		Difference		−18	−20	−7	+0.10
7	ASD	Pre-op.	2.28	161	183	171	0.91
		6 mos postop.	2.14	133	163	116	0.58
		Difference		−28	−20	−55	−0.33
10	ASD	Pre-op.	5.93	183	141	156	0.83
		10 mos postop.	5.92	100	135	71	0.48
		Difference		−83	−6	−85	−0.35
11	ASD	Pre-op.	4.36	144	120	193	1.20
		4 mos postop.	4.11	120	122	120	0.79
		Difference		−24	+2	−73	−0.41
12	ASD	Pre-op.	6.89	149	109	186	1.20
		4 mos postop.	6.60	113	86	139	1.20
		Difference		−36	−23	−47	0
22	VSD	Pre-op.	3.17	132	126	159	1.08
		5 mos postop.	3.21	92	120	91	0.64
		Difference		−40	−6	−68	−0.44
Average		Pre-op.	3.95	144	130	166	1.08
		Postop.	3.91	104	110	110	0.88
		Difference	−0.04	−40	−20	−56	−0.20
Significance of difference of average of pre- and post-operative values† p =				<0.001	<0.025	<0.001	<0.05
			Group II: decreased $\dot{Q}_p$				
25	PS	Pre-op.	2.24	108	125	100	0.73
		5 mos postop.	2.07	100	104	94	0.87
		Difference		−8	−21	−6	+0.14
26	PS	Pre-op.	2.95	78	75	77	0.94
		3 mos. postop.	2.65	71	60	85	1.37
		Difference		−7	−15	+8	+0.43
Average		Pre-op.	2.59	93	100	88	0.83
		Postop.	2.36	86	82	89	1.12
		Difference		−7	−18	+1	+0.29

* Expressed as per cent of the value predicted on the basis of TLC.
† Paired (Student's *t* test) used for calculation of significance.

indicating that the changes, at least in most cases, were apparently reversible. In the two patients operated on for pulmonic stenosis there were no significant changes, although both showed slight decreases in D_{LCO} and D_M and an increase in the R_M/R_c ratio.

DISCUSSION

The significance and limitations of the measurements of D_{LCO}, D_M and V_c have been recently reviewed (17) and the application of the techniques to normal children has been reported (6). The interpretation of diffusion studies involves ques-

tionable assumptions in patients with abnormal air distribution. Abnormal distribution occurs in patients with pulmonary congestion (18); however, on the basis of physical and roentgenological examinations, normal residual volume to TLC ratios, and normal timed vital capacity measurements, no or at most only minor distribution abnormalities were thought to be present in this series of patients with congenital heart disease.

In patients with a left-to-right shunt, varying amounts of blood recirculate through the lungs during the 10-second breath-holding period. This would result in an increased back pressure of CO which would reduce the $D_{L_{CO}}$. If a correction for this factor had been possible, D_M, V_c and the R_M/R_c ratio in patients with left-to-right shunts would have been even greater and the validity of the conclusions of the study would have been strengthened.

The values for the constant θ have been determined by Roughton, Forster and Cander (4, 5) in hemoglobin solutions made from the red cells of normal adults, and there may be factors, unknown at present, which affect these values for the hemoglobin of abnormal individuals. The effect on θ of varying P_{CO_2} is not known, but the addition of as much as 10 per cent CO_2 to the inspired gas produces only minor changes in the 10-second $D_{L_{CO}}$ (19). Furthermore, $D_{L_{CO}}$ does not change significantly with changes in blood pH (20).

One additional limitation in the interpretation of these diffusion studies in patients with congenital heart disease results from the fact that it is not known whether the relationship between the hematocrit of systemic and pulmonary capillary blood is similar in normal subjects and in those with congenital heart disease.

In spite of the above limitations, it is felt that the studies in patients with congenital heart disease are probably comparable to those in normal persons. Since, for the most part, the differences for many of the individual patients and particularly for certain groups are relatively large, it is thought that certain conclusions are warranted.

A number of other authors has reported diffusion studies in patients with congenital heart disease. Auchincloss, Gilbert and Eich (21) and Bedell (22) have found increases in $D_{L_{CO}}$ in several cases of VSD and ASD; normal or only slightly decreased values were found with normal or decreased $\dot{Q}_P$. However, in no cases reported or in the present series has $D_{L_{CO}}$ been decreased enough to suggest even minimal diffusion difficulties. Rankin and Callies (23) found increased $D_{L_{CO}}$, D_M and V_c in 12 patients with conditions associated with increased $\dot{Q}_P$ when pulmonary artery hypertension was absent. Thus, these reports are consistent with the present findings of significant increases in $D_{L_{CO}}$, D_M and V_c in patients with an increased pulmonary artery wedge pressure and $\dot{Q}_P$. The results are in accord with anatomical observations in at least one cause of pulmonary vascular distention, mitral stenosis, in which Parker and Weiss (24) reported pulmonary capillaries which were dilated and possibly increased in number or length or in both. In the relatively few patients studied in the present series who had normal or decreased $\dot{Q}_P$'s, the lowest V_c's were associated with the lowest $\dot{Q}_P$'s.

On the basis of the postoperative studies, it would appear that increases in D_M and V_c, even though presumably present for many years, are usually reversible. In addition, the response to exercise of the patient with a large ASD suggests that, even after years of subjection to increased $\dot{Q}_P$, the capillary bed may increase in size still further.

Mechanisms regulating the size of the pulmonary capillary bed are not entirely clear. However, since no contractile (muscular) elements can be demonstrated in the capillaries, the transmural pressure and the elastic characteristics of the capillary walls must be the locally determining factors. Not only may the pulmonary capillary volume be increased by the opening of capillaries which have been previously closed until their critical opening pressure has been reached (25, 26), but also increases in pressure may further distend capillaries which are already open. In contrast to these local, passive responses of the pulmonary capillaries to changes in pressure, an active regulation of the capillary volume may result from fluctuations in the resistance of the pre- or postcapillary blood vessels.

A number of studies of lung diffusion has attempted to clarify the physiological factors which lead to changes in pulmonary capillary blood volume. In isolated cat lungs Rosenberg and Forster (27) found that increases in intracapillary pres-

sure were associated with increases in V_c as measured by diffusion techniques. In normal subjects and patients with varying hemodynamic conditions a variety of results, often contradictory, has been reported (28–33). The data are difficult to interpret because some of the studies have used the steady state carbon monoxide diffusion technique, and the effect of changes in ventilation per se could not be accurately determined. In addition part or all of the hemodynamic changes have been inferred from other studies of similar situations or from indirect measurements. Nevertheless, the large increases in $D_{L_{CO}}$ and V_c (15, 20, 29) with only minor changes in wedge pressure (34) reported relatively consistently in exercise studies, suggest that there is some active regulation of the resistance of the pre- or post-pulmonary capillary vessels which affects the volume of the pulmonary capillaries.

An irreversible type of change in the resistance of the pre-capillary vessels which may influence V_c is illustrated by patients with pulmonary arterial obstruction. In such patients, although histological examination of the capillaries has indicated no structural changes which might limit distensibility (35), V_c may be normal or reduced even when the pulmonary artery pressure is markedly elevated. Patient 34 would appear to represent such a situation.

In the present study it seems clear that the increase in V_c in most patients with left-to-right shunts is secondary to an increase in $\dot{Q}_P$ and occasionally to an increase in capillary pressure.[7] The importance of capillary pressure as a passive regulating factor is emphasized by the observation that patients with the greatest increase in V_c had also the highest wedge pressures. The failure to find a more exact correlation between wedge pressure levels and V_c is probably based on the variability of the measurements, and lability of circulation and pulmonary function.

Theoretically D_M is dependent upon: *1*) the area of the diffusing surface of the pulmonary capillaries, *2*) the thickness of the membrane, *3*) the specific diffusivity, and *4*) the solubility of CO in the tissues of the pulmonary membrane (37). No information is available on possible changes of the last two factors in health or disease, but great variations are unlikely. The capillary volume/surface area ratio is largely a matter of speculation. The alveolar capillaries have been described as a fine network mesh (38), but detailed information on the morphology in normal and abnormal conditions and at various blood flows and lung distentions is lacking.

In its simplest model the pulmonary capillary network could be considered to be composed of a number of cylindrical segments. In such a model, the ratio of the volume to the area of the curved surface would remain constant when the length of the cylinders increased, and would increase if the radii increased. The latter situation would correspond to an increase of the R_M/R_c ratio. Other possibilities might also be proposed, but too little information on the geometry of the capillaries is available to make further analysis useful.

The R_M/R_c ratio may also increase if the thickness of the pulmonary membrane increases. An increased R_M/R_c ratio has been found in several conditions associated with increased V_c, e.g., in pulmonary congestion (12) and in exercise (15) (Table IV) as well as in our patients with congenital heart disease with or without mitral disease (Table III). Increased or uneven P_{CO} gradients caused by augmented velocity of the capillary blood stream could produce an apparent decrease of D_M in subjects with high $\dot{Q}_P$, but it seems unlikely that this would appreciably affect the results since the P_{CO} equilibration in the pulmonary capillaries is presumably almost instantaneous (17).

Alterations of the volume/surface area ratio and/or increased thickness of the pulmonary capillary membrane might explain the mild increase of the R_M/R_c ratio observed in the majority of the patients. The second possibility is supported by microscopic findings in lungs of patients with mitral stenosis (24). However, further studies are necessary to elucidate the nature of the anatomic and functional changes of the pulmonary capillary membrane in other types of heart disease.

[7] Although it is recognized that the mean pulmonary artery wedge pressure may not be identical to the capillary pressure, the two are closely related when pulmonary arterial obstruction is absent (36). Since only 3 of the 34 patients in the present series had evidence of vascular obstruction, it has been assumed in the discussion which follows that wedge pressure values are representative of capillary pressures.

The postoperative studies indicate that in patients with high $\dot{Q}_P$ the re-establishment of normal hemodynamic conditions is usually followed by a return toward normal of the pulmonary capillary bed. However, since none of the reported patients preoperatively had greatly elevated R_M/R_c ratios, this reversal might not apply to patients with markedly altered pulmonary membranes.

SUMMARY

Studies of total lung diffusing capacity (D_{LCO}), diffusing capacity of the pulmonary membrane (D_M) and pulmonary capillary blood volume (V_c), using the 10-second carbon monoxide technique, have been made in 34 patients with congenital heart disease. Patients with increased pulmonary blood flow ($\dot{Q}_P$) or increased mean pulmonary wedge pressure or both tended to have a significant increase in D_{LCO}, D_M and V_c. Patients with normal or decreased $\dot{Q}_P$ had normal or slightly decreased V_c and D_M values. When patients with increased $\dot{Q}_P$ had surgical correction of their malformation, the D_{LCO}, D_M and V_c usually returned to normal. Exercise studies in one patient with a left-to-right shunt and a high V_c suggested that the pulmonary capillaries may increase in volume still further. The possible mechanisms underlying the changes in diffusion and its components are discussed.

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THE PREPARATION OF CRYSTALLINE BILIRUBIN-C^{14} *

By J. DONALD OSTROW,† LYDIA HAMMAKER AND RUDI SCHMID

(From the Thorndike Memorial Laboratory and the Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)

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Conventional methods for measuring small amounts of bilirubin in biological material, based on the diazo reaction (1–3), have frequently proved unsatisfactory because of relative insensitivity at low pigment concentrations (2, 4–6), problems encountered in standardization (7–9), and loss of pigment through attachment to precipitated protein (1–3). Furthermore, while a small group of tetrapyrrolic chromogens derived from bilirubin can be estimated by their color reaction with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) (10, 11), methods of analysis and quantitation are almost completely lacking for Ehrlich-negative pyrrole compounds (12, 13).

These methodological shortcomings have hindered adequate study of several important aspects of bile pigment metabolism including: the mode of bilirubin transport in the serum at physiological concentrations (14–18), the mechanism of bilirubin exchange between serum and tissue proteins (19, 20), the rate of conversion of bilirubin to non-urobilinoid catabolites (13, 21), and the nature and magnitude of the enterohepatic circulation of bile pigments (22, 23). Clarification of these problems has awaited preparation of radioisotopically labeled bilirubin of high specific activity.

Possible approaches for introducing a radioactive label into the bilirubin molecule include chemical synthesis from opsopyrrolecarboxylic acid-C^{14} (24), tritiation by the Wilzbach technique (25), and biosynthesis from glycine-2-C^{14} (26). Of these, only the last method seemed technically feasible.

The present paper describes a biosynthetic preparation of radiochemically pure bilirubin-C^{14} of high specific activity. It involves administration of glycine-2-C^{14} to rats with enhanced heme production, and subsequent isolation of bilirubin-C^{14} from the bile. Satisfactory yields are achieved by means of a new, relatively simple method for obtaining small amounts of crystalline bilirubin from bile. In connection with these studies, it was necessary to perform preliminary experiments concerning the rate at which bilirubin-C^{14} is excreted in rat bile after injection of glycine-2-C^{14}, and the stability of pure bilirubin in various solutions, both in the dark and on exposure to light. Finally, conventional techniques were adapted for radioassay of bilirubin-C^{14} and for determination of radiochemical purity.

PROCEDURE AND METHODS

Principle. In the biosynthesis of heme, 8 of the 34 carbon atoms of protoporphyrin are derived from the α-carbon of glycine, namely the 4 methene bridge carbon atoms and 1 of the α-carbons of each pyrrole ring (27). In the conversion of heme to bilirubin, 1 methene bridge carbon atom is lost (28), probably in the form of carbon monoxide (29, 30). Thus, after *in vivo* administration of glycine-2-C^{14}, the excreted bilirubin should contain 7 labeled carbon atoms of equal radioactivity (Figure 1).

Studies in man have indicated that, after administration of glycine-N^{15} (31–33) or glycine-2-C^{14} (34), the stercobilin excreted in the feces exhibits two distinct and widely separated peaks of specific activity. The major portion of the radioactive bile pigment is excreted between Days 100 and 140 after administration of the isotope, i.e., at the time when the labeled circulating erythrocytes reach the end of their physiological life span. Another narrower peak, usually of equal height, occurs during the first 20 days after administration of the isotopic glycine. The source of the labeled bile pigment excreted during this "early labeling period" is disputed (21, 31, 35–37) but it appears to be related to the erythropoietic activity of the bone marrow, since, in instances of stimulated erythropoiesis (34, 38) or of qualitatively abnormal red cell formation (32, 39–44), this peak may be greatly magnified.

Circulating erythroyctes in the rat, in contrast to those in man, may not have a uniform life span, but appear in part to be destroyed in random fashion (45, 46). This

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† Postdoctoral Research Fellow, National Institutes of Health, Bethesda, Md.

would indicate that sequestration and destruction of labeled murine red cells may extend over a relatively long period of time, resulting in prolonged low-grade labeling of excreted bile pigments, rather than in the distinct late peak of radioactivity observed in man. Therefore, bile collected during the period of physiological breakdown of rat erythrocytes seemed unsuitable for the purpose of obtaining bilirubin with high specific activity. On the other hand, it was deemed likely that rats would resemble man in excreting highly labeled bile pigments during the first few days following the administration of radioactive glycine, and also that the incorporation of the isotope into bilirubin could be enhanced by stimulating erythropoiesis.

Preparation of the experimental animals. In the preliminary studies, erythroid hyperplasia was induced in adult rats by repeated hemorrhage (47) or by injection of phenylhydrazine (47, 48). Two ml of blood per 100 g body weight was removed by cardiac puncture every other day for 7 days. In other animals phenylhydrazine hydrochloride was administered subcutaneously as a neutralized 2 per cent aqueous solution in an initial dose of 20 mg, followed by 3 doses of 10 mg each, injected every 36 hours. On the fourth day after the last treatment, both groups of animals were given a single injection of 0.2 mc glycine-2-C^{14} and the bile was collected through a catheter in the common bile duct.

Figure 2 shows the results obtained in two male rats of identical weight, each treated by one of the two regimens outlined above. In the rat subjected to repeated hemorrhage, the specific activity of the bilirubin excreted during the first day was 67 per cent higher than that in the animal prepared with phenylhydrazine. On the other hand, the bile of the phenylhydrazine-treated rat contained 40 to 60 mg of labeled bilirubin per 100 ml, while in the bled animal the bilirubin concentration averaged only 5 mg per 100 ml. In both instances, peak specific activity of bilirubin-C^{14} was attained within the first 18 hours after administration of the labeled substance.

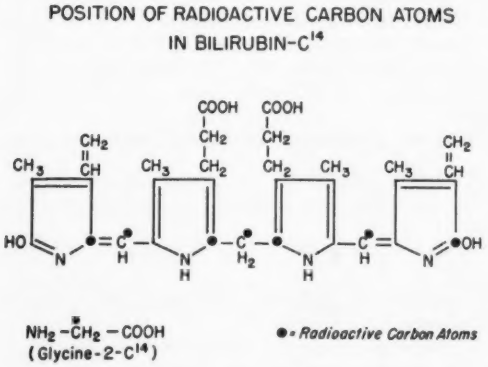

FIG. 1. STRUCTURAL FORMULA OF BILIRUBIN, SHOWING THE POSITIONS OF THE RADIOACTIVE CARBON ATOMS DERIVED FROM GLYCINE-2-C^{14} (27, 28).

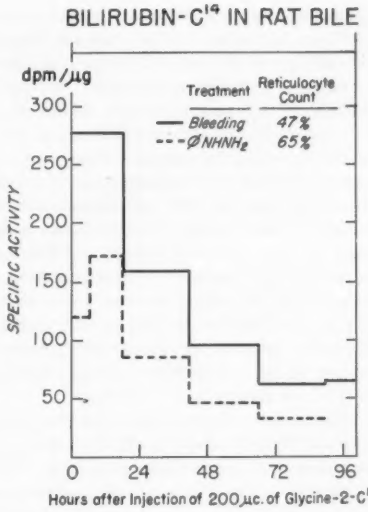

FIG. 2. SPECIFIC ACTIVITY (DISINTEGRATIONS PER MINUTE PER MICROGRAM) OF BILIRUBIN-C^{14} IN FISTULA BILE OF TWO 380 G RATS, AFTER EACH RECEIVED A SINGLE INJECTION OF 0.2 MC GLYCINE-2-C^{14}. Erythropoiesis was stimulated in one animal by repeated bleeding, and in the other with phenylhydrazine. Reticulocyte counts were determined at the time of isotope administration.

Based on these preliminary findings, the experimental animals were thereafter prepared in the following way.

Male Sprague-Dawley rats of 330 to 380 g body weight were given 20 mg phenylhydrazine subcutaneously on Day 1, followed by three injections of 10 mg each on Days 2, 4 and 5. On Days 8, 10, 12 and 14, 2 ml blood per 100 g body weight was removed by cardiac puncture. On Day 15, the rats were anesthetized with Nembutal (pentobarbital) and ether and an external bile fistula was produced. A PE-10 polyethylene catheter [1] (ID 0.011 inches) was anchored in the proximal portion of the common bile duct (49) and the free end was brought out through a stab wound in the right flank. The animals were placed in loose restraining cages and bile drained into plastic tubes containing approximately 50 mg ascorbic acid (50). These tubes were kept on ice in the dark, changed every 6 hours, and stored in the deep freeze at $-20°$ C immediately after each collection period. Bile was collected for 5 to 9 consecutive days, during which time the rats were given access to a drinking solution containing 5 per cent glucose, 0.45 per cent NaCl and 0.05 per cent KCl in 5 per cent (vol/vol) aqueous ethanol. If bile flow fell below 0.7 ml per hour, 4 to 5 ml of an aqueous solution containing 0.45 per cent NaCl and 2.5 per cent glucose was given by subcutaneous injection.

Following successful biliary cannulation, glycine-2-C^{14}

[1] Clay-Adams, Inc., New York 10, N. Y.

with a specific activity of 17.8 mc per mmole [2] was dissolved in 0.85 per cent saline and injected subcutaneously. After an initial dose of 0.4 mc, 0.2 mc was administered every 30 minutes to a total dose of 2.0 mc. Collection of bile for isolation of bilirubin-C^{14} began 60 minutes after the initial injection of radioactive glycine.

Isolation of crystalline bilirubin-C^{14} from bile. Previously reported methods for isolating and crystallizing bilirubin from bile (51–62) did not appear suitable for the present study because they required large quantities of bile, gave a poor yield of pigment of questionable purity, or called for prolonged extraction procedures. The technique to be described obviates these shortcomings, since it is applicable to as little as 0.2 mg bilirubin, consistently yields over 40 per cent of the pigment content of the bile as pure crystalline bilirubin, and can be completed in 3 to 4 hours.

Bile specimens were thawed and ascorbic acid was added to a final concentration of 20 mg per ml. Bilirubin glucuronide was precipitated as the lead salt by addition of one-fifth vol of 5 per cent aqueous lead acetate [$Pb(C_2H_3O_2)_2 \cdot 3H_2O$] (63). The precipitate was packed by brief centrifugation and the supernatant fluid discarded. The surface of the precipitate and the inside of the centrifuge tube were washed once with water and three times with absolute ethanol. The precipitate was then dispersed in 4 vol of absolute ethanol, centrifuged, and the yellow supernatant fluid discarded. After addition of a few milligrams of ascorbic acid, the glucuronide was hydrolyzed by stirring the precipitate with 4 vol of 1 N aqueous sodium hydroxide for 20 minutes at room temperature in the dark (64). Glacial acetic acid was added to bring the pH of the mixture to 5.0, and the unconjugated bilirubin was extracted into 4 vol of chloroform. The precipitate, forming at the interphase, was separated and re-extracted with a 3:1 mixture of chloroform and glacial acetic acid until the extracts were almost colorless. Any precipitate remaining on the surface of the pooled chloroform extracts was then floated off with a small amount of water.

The combined chloroform extracts were washed six times with one-fifth vol of 1 per cent aqueous ascorbic acid, once with an equal volume of 10 per cent saline, and four times with water. The chloroform solution was filtered through Whatman no. 40 paper which had been moistened with chloroform, and the filtrate then evaporated to a small volume in a boiling water bath. After adding one-half vol of methanol and boiling off the remaining chloroform, bilirubin readily crystallized as orange needles. These were separated by centrifugation, washed with 0.5 ml of cold methanol and dried *in vacuo* in the dark over anhydrous $CaSO_4$ and paraffin shavings. For recrystallization, the bilirubin was dissolved in a few milliliters of chloroform and again crystallized as above. With amounts of bilirubin less than 0.3 mg, addition of methanol to the chloroform solution occasionally resulted in formation of a white colloidal precipitate instead of orange crystals. This apparently consisted of aggregates of bilirubin (65–68) which redissolved readily in chloroform.

[2] Lot no. 35-219, New England Nuclear Corporation, Boston, Mass.

TABLE I

Yields of crystalline bilirubin from bile

No. of samples	Source of bile	Bilirubin: Volume	Bilirubin: Concentration	Bilirubin: Content	Yield: Mean	Yield: Range
		ml	*mg%*	*mg*	%	%
15	Rat	0.4–0.6	37–76	0.18–0.39	59.8	(44–93)
9	Rat	2.3–30	4.5–50	0.83–2.32	68.7	(48–82)
4	Human	10–60	45–70	5.5–41.6	68.0	(61–74)

For quantitation, chloroform solutions of the crystals were prepared and the bilirubin concentration measured by the diazo reaction (2) or by direct spectrophotometric determination at 450 mμ (68). Standard curves were prepared for each method with weighed amounts of commercially available bilirubin.[3]

In most instances more than half of the pigment content of the initial bile sample was recovered as crystalline bilirubin (Table I). Similar yields were obtained from human bile collected by drainage of the common bile duct (Table I).

Radioassay of bilirubin-C^{14}. Bilirubin crystals were dissolved in chloroform and 1 to 5 μg was counted at infinite thinness (69) on stainless steel planchets in a windowless gas-flow proportional counter with an efficiency of 58 to 64 per cent. Samples were also counted in a Packard Tri-Carb liquid scintillation spectrometer, using toluene-C^{14} as an internal standard.[4] The chloroform solution, containing 1 to 9 μg of bilirubin-C^{14} was evaporated in low-potassium glass counting vials,[5] the bilirubin was redissolved at 50° C in 0.5 ml 0.5 M Hyamine in methanol [6] (70) and 17 ml of scintillator solution [7] was added (71). Counting efficiency ranged from 44 to 50 per cent. Results were expressed as disintegrations per minute (dpm) per μg bilirubin-C^{14}.

Analysis of crystalline bilirubin-C^{14}. Crystals of bilirubin-C^{14} were dissolved in chloroform or in 0.1 N NaOH and the absorption spectra, as determined in a Beckman DU spectrophotometer, were compared with those reported in the literature (67, 72). Elemental analysis was carried out on 10 mg of thrice recrystallized bilirubin-C^{14}.[8]

Thirty-two samples of bilirubin-C^{14} were recrystal-

[3] Pfanstiehl Laboratories, Inc., Waukegan, Ill.

[4] Lot no. 25-273, New England Nuclear Corp., Boston, Mass.

[5] Catalog no. 6001015, Packard Instrument Co., La Grange, Ill.

[6] Prepared from Hydroxide of Hyamine 10X, Packard Instrument Co., La Grange, Ill.

[7] 2,5 Diphenyloxazole, 4 g per L, and 50 mg per L 1,4 *bis*-2(5-phenyloxazolyl)-benzene, dissolved in dry toluene. Pilot Chemical Company, Watertown, Mass.

[8] Performed by J. F. Alicino, P. O. Box 267, Metuchen, N. J.

lized three to five times each and the specific activity of the radioactive bilirubin was determined on the last two to four crystallizations.

Three individual samples of approximately 0.5 mg of thrice recrystallized bilirubin-C^{14} were subjected to countercurrent distribution, using a manually operated 100-tube glass apparatus.[9] The solvent system consisted of 5 vol freshly distilled chloroform, 3 vol *n*-heptane purified by passing through silica gel, and 4 vol dry propylene glycol. The system was equilibrated for 24 hours in a dry, dark glass bottle, forming an upper phase consisting mainly of *n*-heptane and chloroform and a lower phase of propylene glycol and chloroform. During the distribution, the apparatus was protected from direct sunlight. After 50 transfers, the 20-ml solvent system (10 ml upper; 10 ml lower) in each of the tubes was extracted with 0.5 vol of distilled water to remove the propylene glycol. In preliminary studies with bilirubin-C^{14}, it was ascertained that the colorless propylene glycol-water extract contained no significant radioactivity. An aliquot of the remaining chloroform-*n*-heptane mixture was used for determination of pigment concentration by comparing the optical density at 455 mμ with a standard curve prepared with commercially available bilirubin. Another aliquot was evaporated in low-potassium glass counting vials in a boiling water bath, the *n*-heptane being removed as an azeotrope with ethanol. The residue was dissolved in Hyamine-methanol and counted in a liquid scintillation spectrometer as described above. From the data obtained, the partition coefficient, K, of bilirubin in this solvent system was calculated to be 3.0 and the theoretical distribution curve was constructed (*73*). The specific activity data were treated mathematically as outlined by Baggett and Engel (*74*).

Studies of the stability of bilirubin solutions. Previous reports have indicated that in organic or in alkaline aqueous solutions bilirubin is unstable and may decompose or become oxidized to biliverdin (*50, 72, 75, 76*), particularly if exposed to light (*7*). It was therefore necessary to obtain detailed information on the behavior of bilirubin in those media used during the isolation, crystallization, radiochemical analysis and quantitation of bilirubin-C^{14}. In addition, solutions of bilirubin in 0.1 N NH_4OH or 0.1 N NaOH were investigated, because these are commonly used vehicles for introducing bilirubin into experimental biological systems.

Approximately 1.0 mg recrystallized bilirubin was dissolved in 100 ml of each of the following media: 0.1 N NaOH, 0.1 N NH_4OH, benzene, chloroform, or chloroform-methanol mixtures in the proportions of 1:7, 1:1 and 7:1. Half of the solution was kept in the dark, while the Erlenmeyer flask containing the remainder of the solution was exposed to an unfiltered G. E. mercury lamp at a distance of 25 cm. Immediately after dissolving the bilirubin and at intervals thereafter, aliquots from the light-protected and light-exposed solutions were removed for spectral analysis in the range from 325 to 700 mμ, for the diazo reaction and for the pentdyopent reaction. The concentrations of bilirubin in organic solvents were determined by the Malloy-Evelyn method (*2*), whereas for aqueous alkaline solutions, the diazo reagent was prepared in 0.6 N HCl and the azopigment estimated at 560 mμ. For the pentdyopent reaction, 1.5 ml of aqueous alkaline bilirubin solution was mixed with 1.5 ml of 2.0 N NaOH and 1 to 2 mg of sodium hydrosulfite ($Na_2S_2O_4$). After heating in a boiling water bath for 5 minutes, the optical density was determined at 525 and 530 mμ (*77*) against a blank of 1.0 N NaOH containing a comparable amount of hydrosulfite. With bilirubin in organic solvents, 1.5 ml of the solution was extracted with 3.0 ml of 1 N NaOH, and the pentdyopent reaction was performed on the undiluted extract.

A sample of crystalline bilirubin-C^{14} dissolved in 0.1 N NH_4OH was exposed to a G. E. mercury lamp for 5 hours, resulting in a virtually colorless solution giving a negative diazo and a strongly positive pentdyopent reaction. The water and ammonia were evaporated *in vacuo* and the residue was dissolved in propylene glycol and subjected to countercurrent distribution in the solvent system described above.

Freshly collected rat bile was stored in plastic tubes in the deep freeze at −20° C. At intervals of several weeks to several months the bile was thawed and aliquots were removed for bilirubin determination (*2*). Radiochemically pure crystalline bilirubin-C^{14} was stored for 1 year in a dark vacuum desiccator containing $CaSO_4$ and paraffin shavings. The specific activity of the radioactive bilirubin was determined before and after the storage period.

RESULTS

Yield and specific activity of bilirubin-C^{14}. Rats prepared as described maintained a bile flow of 0.7 to 1.3 ml per hour with biliary bilirubin concentrations ranging from 8 to 13 mg per 100 ml. This permitted isolation of 1.2 to 1.9 mg of twice recrystallized bilirubin-C^{14} per day. Bilirubin-C^{14} excreted during the first 24 hour period following the initial injections of labeled glycine exhibited a mean specific activity of 2,500 to 3,800 dpm per μg or 0.66 to 1.0 mc per mmole (Figure 3). During the second and third 24-hour collection periods, the specific activity decreased to about one-half and one-third, respectively, of these peak values, and thereafter levelled off at approximately one-fourth of this value (Figure 3). From the preliminary studies (Figure 2) it is apparent that maximal specific activity of bilirubin-C^{14} was reached between 6 and 18 hours after isotope administration.

[9] H. O. Post Scientific Instrument Company. The authors are grateful to Drs. I. Weliky and L. L. Engel, Huntington Laboratory, Massachusetts General Hospital, Boston, for permitting the use of their equipment and for their help in carrying out these analyses.

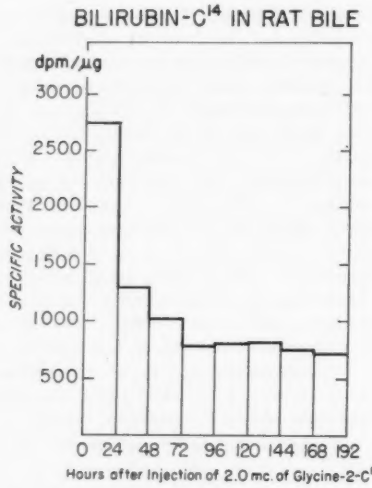

FIG. 3. SPECIFIC ACTIVITY OF BILIRUBIN-C^{14} IN FISTULA BILE OF A 350 G RAT AFTER INJECTION OF 2.0 MC GLYCINE-2-C^{14} OVER A PERIOD OF 4.5 HOURS. The animal was prepared with both phenylhydrazine and bleeding as outlined under Procedure and Methods.

Radiochemical purity of bilirubin-C^{14}. Solutions of crystalline bilirubin-C^{14} in chloroform and in 0.1 N NaOH showed absorption spectra corresponding to those previously reported for bilirubin (67, 72) with maxima at 450 and 420 mμ, respectively. Elemental analysis agreed closely with the calculated composition of bilirubin (78)—calculated: C, 67.79; H, 6.21; N, 9.58; found: C, 67.70; H, 6.29; N, 9.39. Within the limits of the experimental methods employed, the specific activity of each sample of bilirubin-C^{14} remained constant after the first recrystallization.

Analysis of the countercurrent distribution of crystalline bilirubin-C^{14} (Figure 4) yielded curves for radioactivity and for bilirubin closely corresponding with each other and with the theoretical distribution curve for a partition coefficient, K 3.0 (73). Secondary peaks due to radioactive impurities were not observed. When the logarithms of the specific activity in tubes 32 to 43 were plotted (Figure 4), no significant regression existed ($p > 0.2$), indicating that over the span of $\pm 2\theta$ (74), no separation of label and bilirubin had occurred.

Deterioration of bilirubin in aqueous alkaline solution. Solutions of crystalline bilirubin in 0.1 N NaOH or 0.1 N NH_4OH kept in the dark, showed slow deterioration of the pigment as indicated both by progressive reduction in optical density in the 420 to 440 mμ band and diminished formation of the diazo derivative (Figure 5). With a 1 mg per 100 ml solution of bilirubin in 0.1 N NH_4OH, optical density at 440 mμ decreased by 20 per cent in 2 hours and by 60 per cent in 20 hours, associated with progressive fad-

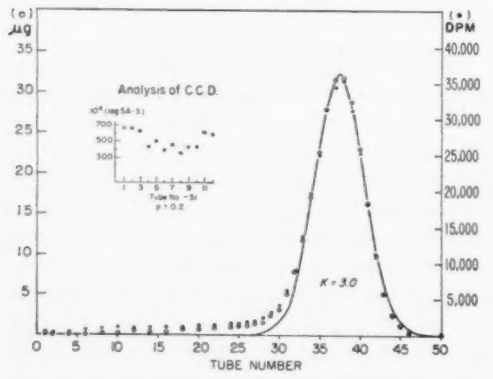

FIG. 4. FIFTY-TRANSFER COUNTERCURRENT DISTRIBUTION OF CRYSTALLINE BILIRUBIN-C^{14}, USING AS SOLVENT SYSTEM CHLOROFORM: *n*-HEPTANE: PROPYLENE GLYCOL (5:3:4). Open circles indicate bilirubin content, closed circles radioactivity, and the solid line the theoretical distribution curve for K 3.0. The slope, calculated from the logarithms of the specific activity for tubes 32 to 43 (insert), was not significantly different from zero ($p > 0.2$).

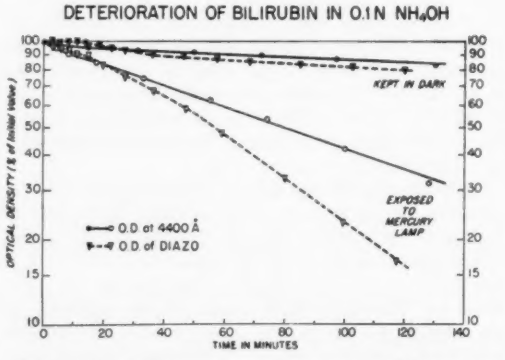
DETERIORATION OF BILIRUBIN IN 0.1 N NH4OH
KEPT IN DARK
EXPOSED TO MERCURY LAMP
O.D. at 4400 Å
O.D. of DIAZO
OPTICAL DENSITY (% of Initial Value)
TIME IN MINUTES

FIG. 5. DETERIORATION OF BILIRUBIN IN 1.0 MG PER 100 ML SOLUTION IN 0.1 N NH_4OH, KEPT IN THE DARK OR EXPOSED TO LIGHT. The values obtained for optical density at 4,400 Å (solid line) and with the diazo reaction (dotted line) are plotted as percentage of the values at zero time.

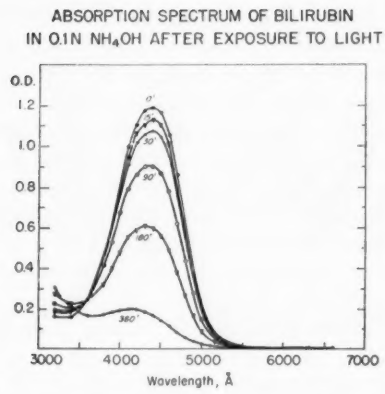

FIG. 6. SERIAL ABSORPTION SPECTRA OBTAINED DURING EXPOSURE OF A 1.0 MG PER 100 ML BILIRUBIN SOLUTION IN 0.1 N NH_4OH TO A G. E. MERCURY LAMP AT A DISTANCE OF 25 CM. The progressive decrease in optical density in the 4,400 Å region was associated with fading of the yellow color. The pentdyopent reaction was strongly positive after 2 and 4 hours of exposure, and weakly positive after 7 hours.

ing of the yellow color. Moreover, the initial absorption maximum at 440 mμ shifted to a peak at 420 mμ after 20 hours. No green or blue discoloration was observed at any time nor did significant absorption appear in the 600 to 700 mμ band. However, a positive pentdyopent reaction, with peak absorption between 525 and 530 mμ, first developed after 2 hours of storage and steadily increased to a maximum at 25 hours.

Exposure of these same aqueous alkaline solutions of bilirubin to intense illumination resulted in deterioration which was qualitatively similar, but occurred at an accelerated rate (Figures 5 and 6). Under these conditions the solutions became virtually colorless in 6 to 7 hours, with the decrease in concentration of diazo-reacting pigment proceeding more rapidly than the reduction in optical density between 420 and 440 mμ (Figure 5). As in the dark, no significant absorption in the 600 to 700 mμ band was observed (Figure 6). Moreover, the pentdyopent reaction became strongly positive after 2 hours, but was only weakly positive at 7 hours and negative thereafter.

When a sample of bilirubin-C^{14} dissolved in 0.1 N NH_4OH was illuminated for 5 hours and the resulting colorless solution evaporated *in vacuo*, the countercurrent distribution of the residue differed strikingly from that found with bilirubin-C^{14}. Virtually all radioactivity was present in the first four tubes, while in tubes 35 to 40, where bilirubin ordinarily accumulates, no significant radioactivity could be detected. Furthermore, at the completion of the countercurrent distribution all radioactivity in the collecting tubes remained in the aqueous propylene glycol extract, while none was present in the chloroform-heptane mixture.

Deterioration of bilirubin in organic solvents. Solutions of bilirubin in benzene, chloroform, or chloroform containing up to 50 per cent (vol/vol) methanol were stable for at least 2 hours if kept in the dark or in ordinary room light protected from direct sunlight (Figure 7). On the other hand, direct illumination by a mercury lamp resulted in rapid decomposition of the bilirubin as indicated by concomitant reduction in optical density at 450 mμ and in ability to form a diazo derivative (Figure 7). In contrast with the findings in aqueous alkaline solution, the original yellow color changed to an olive green exhibiting absorption maxima at 380 and 650 mμ, typical of biliverdin (79, 80). After 60 to 90 minutes of exposure to the mercury lamp, this green color gradually faded, the solution eventually becoming colorless and strongly pentdyopent-positive.

Bilirubin dissolved in a 7 : 1 (vol/vol) mixture of methanol and chloroform decomposed in a fashion similar to that observed with aqueous alkaline solutions, except that, on exposure to a mercury lamp, transient formation of small

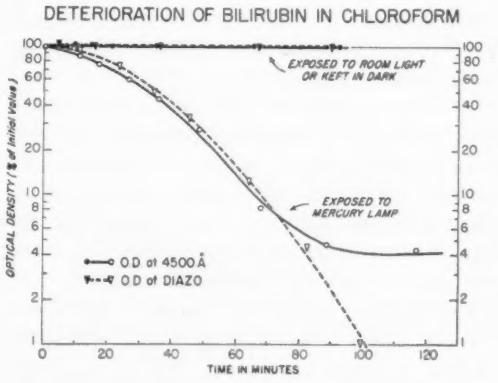

FIG. 7. BEHAVIOR OF A 2.0 MG PER 100 ML SOLUTION OF BILIRUBIN IN CHLOROFORM IN THE DARK, IN ROOM LIGHT, AND ON INTENSE ILLUMINATION. Data are plotted as in Figure 5.

amounts of biliverdin was observed during the initial 2 hours of illumination.

Storage of rat bile in deep freeze at − 20° C, and of crystalline bilirubin-C^{14} in a light-protected desiccator did not result in significant loss of diazo-reacting pigment, nor in change of specific activity of the radioactive bilirubin.

DISCUSSION

The above biosynthetic method permits preparation of several milligrams of radiochemically pure bilirubin-C^{14} with a specific activity adequate for most experimental work. By selecting a larger animal than the rat, or by administering more than 2 mc of glycine-2-C^{14}, one could presumably obtain larger quantities of radioactive bilirubin or achieve still higher specific activities, but, for technical and economic reasons, the procedure as outlined appeared to be most advantageous.

Using the isolation procedure described, crystalline bilirubin has been prepared from both rat and human fistula bile with relative yields well above those obtained by previously reported methods (51–62). This improvement results largely from the following modifications of technique: quantitative precipitation of bilirubin glucuronide from bile as the lead salt (63); rapid and complete hydrolysis of the glucuronide at alkaline pH (64); repeated addition of ascorbic acid to minimize oxidation of bilirubin in aqueous solution (50), and crystallization of the pigment from methanol, in which it is much less soluble than in chloroform (68). An additional factor contributing to the increased yield probably is the relatively short duration of the isolation procedure, resulting in less deterioration of pigment.

Elemental analysis and absorption characteristics in the visible range indicated that the substance crystallized from the bile was identical with bilirubin. The constant specific activity on repeated recrystallization and the result of the countercurrent distribution demonstrated that the compound was of high radiochemical purity. In crystalline form, bilirubin-C^{14} could be stored in a light-protected desiccator for at least a year without decomposition.

As seen in Figure 2, bilirubin-C^{14} appeared in the bile within 6 hours following administration of the labeled glycine and highest specific activity of the excreted pigment was reached between 6 and 18 hours. For comparison of these observations with similar experiments in man (31, 34, 38) allowance must be made for the fact that, in the human, isotope concentration was determined in stercobilin excreted in the feces rather than in biliary bilirubin. After making appropriate adjustments for the delay in pigment excretion and the dilution of the label caused by intestinal transit and enterohepatic recirculation (22, 23), the results of the murine and the human studies are in close agreement. The present observations do not further clarify previously expressed concepts regarding the origin of bile pigments excreted during the "early-labeling period" (21, 31, 34–38).

The lower isotope concentration found in the bilirubin from phenylhydrazine-treated rats (Figure 2) resulted from dilution of excreted bilirubin-C^{14} with increased amounts of nonlabeled pigment (81) derived from continued destruction of erythrocytes which had previously been injured by the toxin (82). In the definitive procedure adopted for preparation of the experimental animals, erythropoiesis was initially stimulated with phenylhydrazine and the erythropoietic hyperactivity then maintained by repeated hemorrhage. This permitted removal of virtually all injured erythrocytes before the labeled glycine was administered, both by splenic sequestration (83) and by bleeding. Consequently, excretion of nonlabeled pigment was substantially reduced, resulting in higher specific activity of the isolated radioactive bilirubin (Figure 3).

In confirmation of previous reports (50, 72, 75, 84–86), aqueous alkaline solutions of unconjugated bilirubin were found to be unstable, even when protected from light. Contrary to a widely held belief (75, 87), however, the major oxidation product(s) was not biliverdin, but a diazo-negative yellow intermediate(s) (88, 89). This yellow color gradually faded and the solution became colorless. Concomitantly, a strongly positive pentdyopent reaction developed, which indicated that some of the bilirubin had been converted to dioxy-dipyrrylmethanes or propentdyopent (Figure 8) (88, 89). Unlike bilirubin (90), these colorless breakdown products are very soluble in water (86, 88), as illustrated by the appearance of most of the radioactivity in the propylene glycol-water phase after countercurrent distribution of the products obtained by illumination of

PROPENTDYOPENT

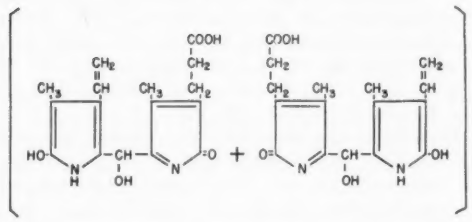

FIG. 8. STRUCTURAL FORMULAE OF THE TWO ISOMERIC FORMS OF PROPENTDYOPENT ACCORDING TO SIEDEL (13).

bilirubin-C^{14} in dilute ammonia. Decomposition of bilirubin in aqueous alkaline solution was qualitatively similar in the dark and in the light, but occurred far more rapidly under the latter condition. These findings re-emphasize that, in order to prevent decay, exposure of bilirubin to aqueous alkali should be avoided. When this is not feasible, decomposition of the pigment should be minimized by addition of ascorbic acid, by exclusion of light, and by restriction of duration of exposure to a high pH.

If protected from intense illumination, bilirubin was stable for at least a few hours in chloroform (7, 87) or benzene. This permitted preparation of the appropriate organic solutions for crystallization, countercurrent distribution and quantitation, without significant decomposition. By contrast, in methanolic solution containing only small amounts of chloroform, bilirubin was unstable even in the dark (7, 91). Therefore, after crystallizing bilirubin from methanol, it is desirable to separate quickly the formed crystals from the mother liquor by centrifugation.

Bilirubin in organic solvents, when exposed to direct illumination, deteriorated very rapidly but, in contrast to aqueous alkaline solutions, some biliverdin could be detected. Conceivably, biliverdin formation is also the first oxidation step in dilute alkali, but this phase may be so transient as to escape spectrophotometric detection. As in aqueous alkali, illuminated organic solutions of bilirubin eventually became colorless, diazo-negative, and pentdyopent-positive.

Formation of propentdyopent (Figure 8), which yields red pentdyopent on reduction in alkali, has been shown to be a group reaction given by a variety of tetrapyrroles on treatment with hydrogen peroxide (13, 77). In the present studies, however, dioxy-dipyrrylmethanes were formed in aqueous alkaline (88, 89) and organic solutions of bilirubin in the absence of added hydrogen peroxide or other oxidizing agents (77). This suggests the possibility that a similar breakdown of bilirubin to dipyrrolic compounds may occur in the organism (77, 92) or in the excreta (93, 94), particularly after exposure to light (95, 96).

SUMMARY

1. A procedure is described for preparing radiochemically pure bilirubin-C^{14} which involves administration of glycine-2-C^{14} to rats with stimulated erythropoiesis, and subsequent isolation of the radioactive pigment from fistula bile.

2. A new and rapid method is presented for obtaining crystalline bilirubin from bile with yields consistently in excess of 40 per cent.

3. With these techniques, crystalline bilirubin-C^{14} was prepared, with a specific activity ranging from 0.66 to 1.0 mc per mmole. Radiochemical purity of the labeled pigment was demonstrated by spectrophotometry, elemental analysis, recrystallization to constant specific activity, and countercurrent distribution.

4. In rats injected with glycine-2-C^{14}, the "early labeling peak" of the excreted radioactive bile pigment was shown to occur between 6 and 18 hours following administration of the isotope.

5. Bilirubin dissolved in benzene, chloroform or in chloroform containing up to 50 per cent methanol, was stable when kept in the dark but rapidly deteriorated on illumination. In alkaline solution bilirubin was unstable even in the dark. The breakdown products formed included yellow diazo-negative pigments and colorless propentdyopent.

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TOTAL NONDIALYZABLE SOLIDS (TNDS) IN HUMAN URINE. IX. IMMUNOCHEMICAL STUDIES OF THE R–1 "UROMUCOID" FRACTION *

By WILLIAM H. BOYCE, J. STANTON KING, Jr.† and MARVEL L. FIELDEN

(From the Departments of Urology and Biochemistry, The Bowman Gray School of Medicine of Wake Forest College, Winston-Salem, N. C.)

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A method has been described for separation of the nondialyzable solids of normal urine into three major fractions (1). The R-1 fraction is composed of the portion which is not filtrable through collodion membranes of average pore diameter 20 mμ, and which is insoluble in veronal buffer of pH 8.6 and ionic strength 0.1. A mean excretion rate of 90 mg per 24 hours (range, 51 to 168 mg per 24 hours) has been established for both male and female adults. Attempts to separate the R-1 into subfractions by electrophoresis, various solvents, and ultracentrifugation have failed to provide subfractions which were distinctly different in composition from the original material. The present paper reports a further characterization of the R-1 mucosubstance by immunological methods.

MATERIALS AND METHODS

The R-1 fraction of normal human urine was recovered, as previously described (1, 2), by veronal buffer (pH 8.6, ionic strength 0.1) extraction of the nonultrafiltrable solids of dialyzed urine. The fraction was suspended in distilled water and centrifuged at 40,000 G for 30 minutes, leaving a supernatant colorless opalescent gel or solution, or both, of approximately 5 mg per ml, which was used in the following experiments. The analyses of the centrifugate and supernate are given in Table I; the brown pellet is composed at least partly of cellular detritus and some inorganic crystalline material. The supernatant colloid migrated electrophoretically as a single hypersharp peak of ascending mobility 9.6×10^{-5} $cm^2 \cdot sec^{-1} \cdot volt^{-1}$ in veronal buffer of pH 8.18 and ionic strength 0.01 at a potential gradient of 5.8 v per cm. A frozen but unlyophilized sample of R-1 in water (5 mg per ml) was ultracentrifuged by M. C. Davies, Lederle Laboratories. A single hypersharp gradient of rapidly sedimenting material was present with a barely detectable trace of a slower moving component. Lyophilization increased the relative quantity of the slower component.

Freshly prepared unlyophilized material had an absorption maximum in the ultraviolet at 277 mμ, and solubility properties like those of the urinary mucoprotein of Tamm and Horsfall (3), including precipitability with cetyltrimethylammonium bromide (CTAB) (4). At least 96 per cent of the hexosamine emerged from a Gardell column (5) at the same effluent volume as glucosamine, the rest appearing at the same volume as galactosamine.

Preparation of antibodies. Aqueous suspensions of freshly prepared R-1 were mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories); the final suspension contained 8 mg of the antigen per ml. This suspension was injected into the foot-pads of adult rabbits so that each animal received 64 μg of antigen nitrogen in each of four feet. Injections were repeated at intervals of 1, 3 and 6 weeks. Intravenous challenge was avoided, since it regularly resulted in anaphylactic death. There was significant antibody production at 10 days. The high titers of antibody obtained at 8 weeks have been maintained as long as 6 months without challenge. Six rabbits were used in each of four groups for antibody production. Serum was pooled and fractionated by the first steps of Cohn's method 10 (2). The entire antibody was found in the fraction corresponding to Cohn fractions I, II, III (γ-globulins). The titers were sufficiently high that very satisfactory qualitative reaction, with the gel diffusion technique, was obtained with unfractionated rabbit antiserum. Antisera of identical

TABLE I

Analysis of lyophilized samples of supernatant gel and liquid phase after ultracentrifugation of a specimen of fraction R-1

	Hexosamine*	Hexose†		Fucose	Sialic acid	N
		Anthrone	Orcinol			
Pellet‡	2.9	16.6	8.7	2.6	<1	7.45
Gel§	6.5	14.4	7.3	1.5	<1	9.73
Liquid§	6.9	15.4	7.3	1.5	<1	9.84

* As base.
† Standard: equal weights galactose and mannose. Figures include fucose.
‡ Approximately 25 per cent of fraction R-1 by dry weight.
§ Used as antigen.

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†Helen Hay Whitney Foundation Research Fellow.

properties were produced in two rabbits by omitting the Freund's adjuvant, but required two to three times the above number of challenge doses.

Rabbit antihuman serum was prepared in a similar manner, using fresh human serum as the antigen.

Preparation of test antigens. The fractions of total nondialyzable solids of normal urine were prepared as previously described (1, 2). Samples of Tamm and Horsfall urinary mucoprotein (3), Di Ferrante-Rich mucosubstance (6), Maxfield mucoprotein (7) and Anderson-Maclagan material (8) were prepared by the methods of the respective investigators.

Human renal tissue, removed by surgical biopsy of 8 kidneys requiring heminephrectomy (calculi or hydronephrosis), was frozen at −18° C. Twenty-four hours later the tissue was thawed and separated into bits of cortex, medulla, renal pelvis and transitional epithelium stripped from the renal pelvis. The samples were ground in sterile tissue grinders to prepare a slurry of approximately 1 part tissue to 5 parts distilled water. Biopsies of skin, subcutaneous fat, fascia and striated muscle from the abdominal wall were prepared in similar fashion. Virus hemagglutination-inhibitory mucoprotein of saliva was prepared by the method of Marmion, Curtain and Pye (9). Whole saliva was also utilized as an antigen.

Erythrocyte membranes were prepared from fresh whole blood (disodium ethylenediamine tetraacetate anticoagulant). After centrifuging to separate the plasma, the erythrocytes were washed three times in 0.85 per cent saline and frozen at −18° C for 24 hours. The erythrocytes were thawed and washed three times with 1 M NaCl and the membranes recovered by ultracentrifugation at 10,000 G, solubilized by cold homogenization in

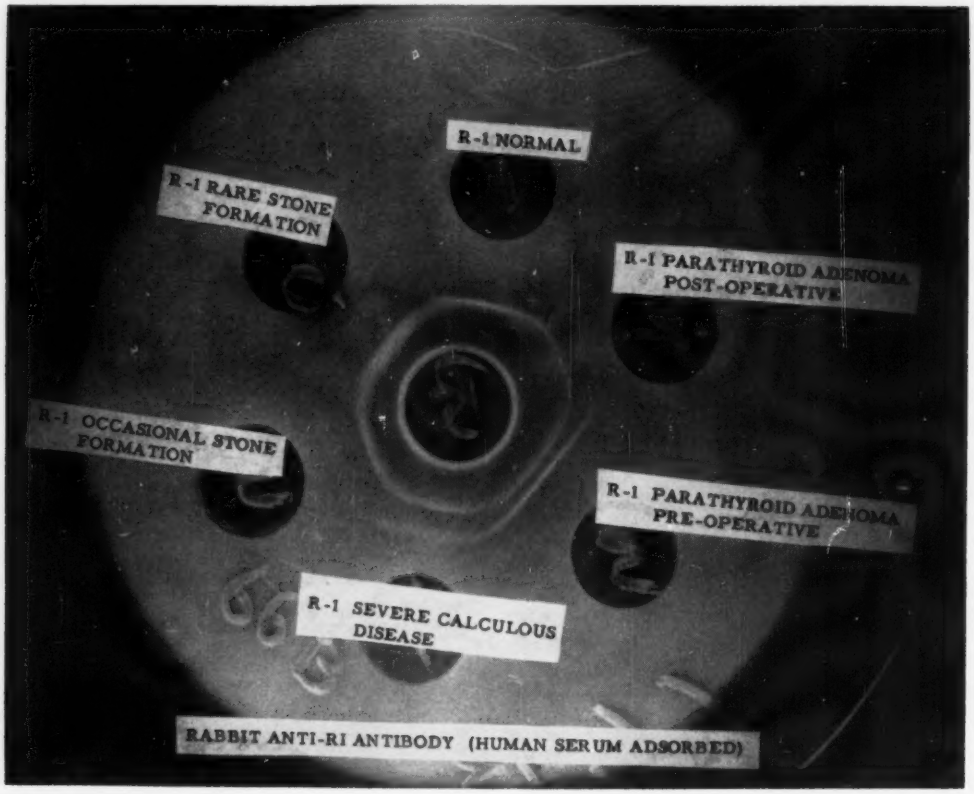
R-1 NORMAL
R-1 RARE STONE FORMATION
R-1 PARATHYROID ADENOMA POST-OPERATIVE
R-1 OCCASIONAL STONE FORMATION
R-1 PARATHYROID ADENOMA PRE-OPERATIVE
R-1 SEVERE CALCULOUS DISEASE
RABBIT ANTI-R1 ANTIBODY (HUMAN SERUM ADSORBED)

FIG. 1. REACTION OF IDENTITY BETWEEN R-1 MUCOID FROM NORMAL URINE AND FROM PATIENTS WITH RENAL CALCULI. Anti-R-1 antibody in center well contains 480 μg of nitrogen, antigens in peripheral wells contain 60 to 80 μg of nitrogen. The peripheral wells are numbered clockwise, with no. 1 at 12 o'clock. The significance of the two zones of precipitation in wells 3 and 4 is unknown. A patient with a single parathyroid adenoma and sterile urine had 127 mg of R-1 per 24 hour urine volume preoperatively and 109 mg between 48 and 72 hours after operation. The RS-1 excretion was 149 mg per 24 hours preoperatively, of which 100 mg had α_1-globulin mobility at pH 8.6. In the period 48 to 72 hours after operation, the RS-1 excretion was 33 mg of which 3.7 mg was α_1 component.

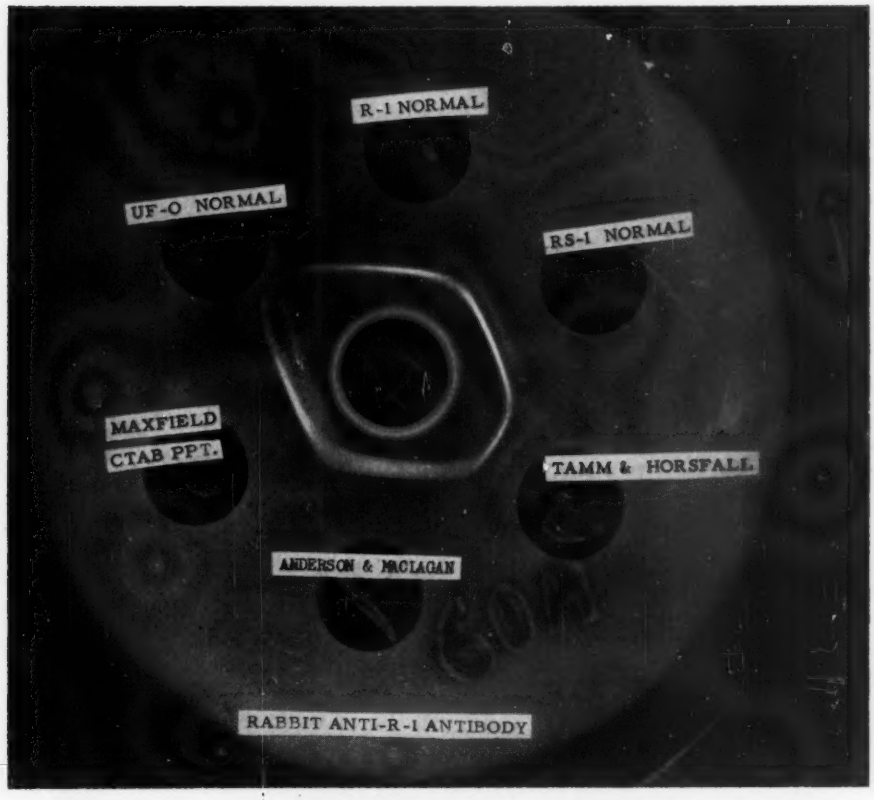

FIG. 2. REACTION OF IDENTITY BETWEEN R-1 AND MUCOSUBSTANCES OF NORMAL URINE RECOVERED BY VARIOUS TECHNIQUES. All test substances were lyophilized and each peripheral well contains approximately 1 mg of dry weight in 0.4 ml of distilled water. Freshly prepared unlyophilized preparations give similar precipitin reactions. The Tamm and Horsfall substance is the total precipitate from 0.58 M NaCl-treated urine, and hence contains the portions soluble and insoluble in phosphate buffer. Two zones of precipitation commonly occur with this "crude" preparation. The UF-O does not give precipitin reactions with anti-R-1 antibody.

distilled water or by suspension in 0.17 M urea, followed by dialysis against physiological saline to remove the urea. Samples of fresh and lyophilized R-1 were carried through all steps of this procedure without loss of the immune response to rabbit anti-R-1 antibody.

Immediately after removal, 22 calcigerous calculi were scrubbed with a sterile brush to remove surface debris and placed in cellophane bags in a 5 per cent solution of ethylenediamine tetraacetate, pH 7.8. Dialysis with mechanical agitation at 3° C removed the crystalline material in 5 to 12 days. The matrix remaining within the cellophane was dialyzed against distilled water and concentrated by suspending the cellophane containers in air currents at 3° C or by lyophilization. As a control, samples of freshly recovered R-1 were placed in cellophane bags and concomitantly carried through all steps of the preparation of the stone matrix. Preparations of whole bone matrix and collagen-free bone matrix were prepared as previously described (10).

Human plasma dilutions were made with physiological saline. Complement was removed by incubation at 56° C for 30 minutes and by storing at 3° C for 5 days. Concentrates of whole plasma proteins were prepared by dialyzing fresh plasma against distilled water and subsequent lyophilization. The dry proteins were taken up in physiological saline to give concentrations up to ten times those of normal serum. These lyophilized preparations retained their serological reactivity with rabbit antihuman serum protein antibody, giving six or more zones of precipitation in the gel diffusion tests.

Immunochemical methods. The technique for determination of the optimal proportions of antibody and antigen followed those of Dean and Webb (11). The technique of precipitation reactions in gels was that of Ouchterlony

(12). Aseptic technique was maintained at all stages of the study.

RESULTS

Presence of R-1 in various fractions of urinary nondialyzable solids. Aqueous suspensions of R-1 antigen in concentrations of 0.05 to 10 mg per ml gave a single distinct zone of precipitation in gel diffusion studies with the rabbit anti-R-1 antibody. This represents a range of approximately 20-fold from the optimal concentrations for serological reactivity as determined by the tube precipitation technique. A "reaction of identity" was obtained with R-1 from normal male and female subjects and from patients with renal calculi, pyelonephritis, Beck's sarcoidosis, multiple myeloma, and lupus erythematosus (Figure 1).

The antigenic reaction of R-1 to anti-R-1 antibody was well preserved after storage as a lyophilized powder for 5 years, dialysis against 10 per cent EDTA at pH 7.8 for 2 weeks and against saturated urea for 1 hour, or heating at 100° C for 1 hour with 0.17 M urea. Two zones of precipitation in gel were usually present in these various preparations of R-1. The more diffusible component invariably gave a reaction of identity with the freshly prepared R-1 antigen. The serological reactivity was still present in a solution of R-1 made to pH 2.5 with HCl, allowed to stand 5 hours, then dialyzed against water. After adjustment to pH 11.7 with NaOH, standing and dialysis, the serological reactivity with rabbit anti-R-1 antibody was not observed. Fresh human serum as a vehicle for suspensions of lyophilized R-1 (50 μg to 5 mg per ml) did not interfere with the precipitin reactions of R-1 to anti-R-1 antibody. Toluidine blue-O stained R-1 orthochromatically, but did not interfere with the serological reactivity.

The preparations of Tamm and Horsfall uri-

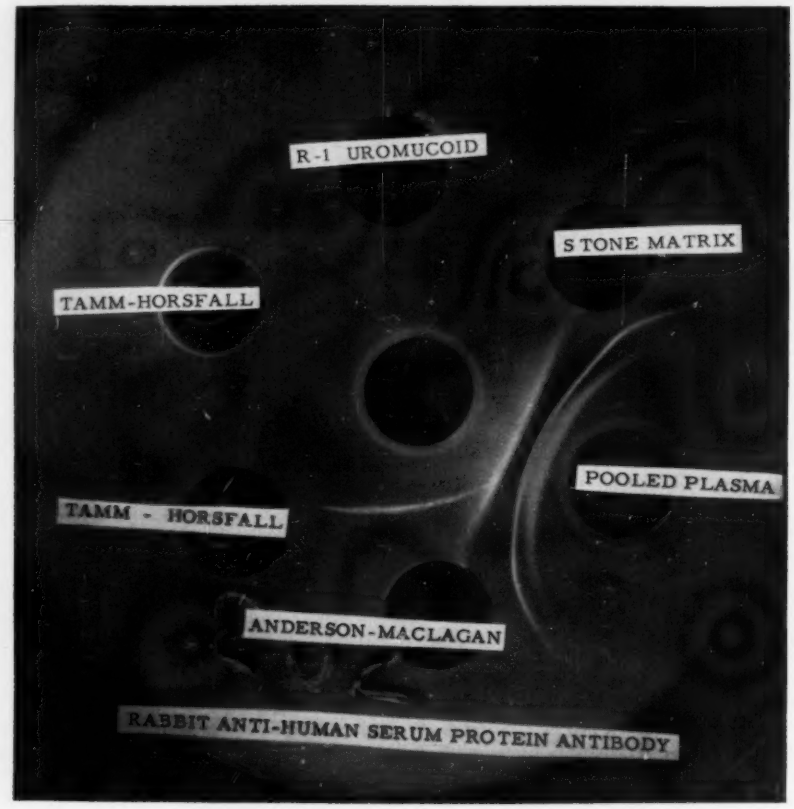

FIG. 3. ABSENCE OF PRECIPITIN REACTION BY R-1 AND TAMM AND HORSFALL SUBSTANCES MATCHED WITH RABBIT ANTIHUMAN SERUM PROTEIN ANTIBODY.

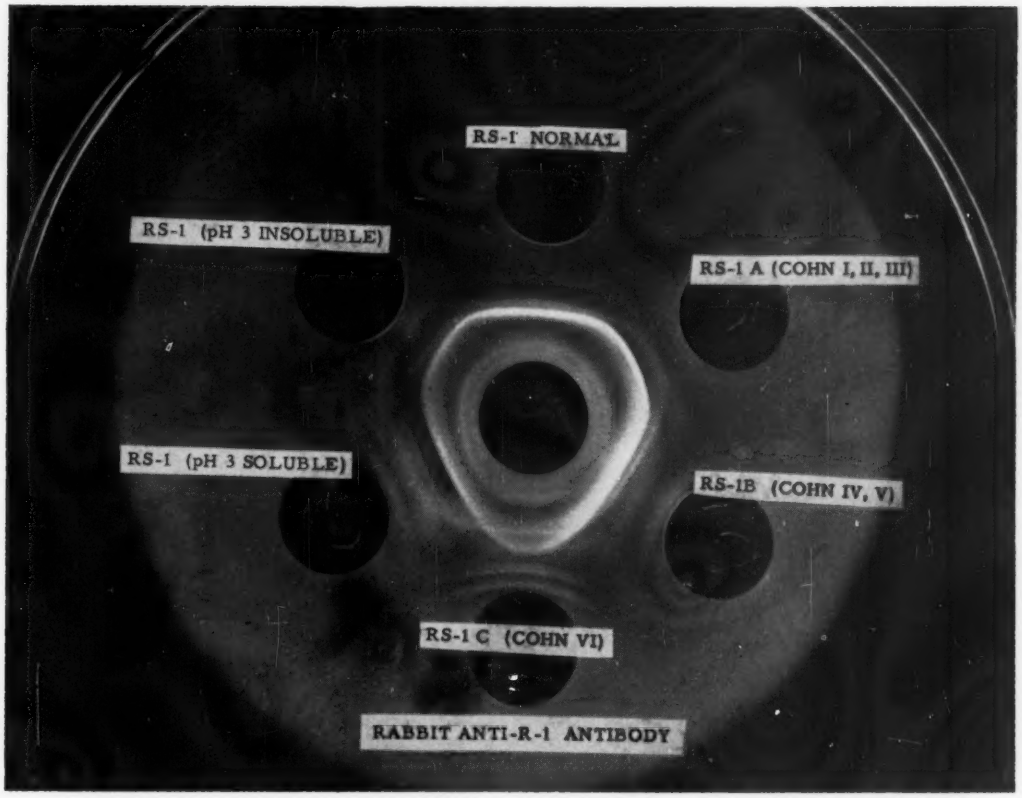

FIG. 4. MULTIPLICITY OF PRECIPITIN ZONES APPEARING WITH ANTI-R-1 ANTIBODY WHEN RS-1 IS TREATED WITH DILUTE ETHANOL, ZINC ACETATE AND DILUTE ACETIC ACID. The subfractions of RS-1 were prepared from the same pool of normal RS-1. Peripheral well 1 contains 0.5 mg of original material; other wells contain approximately 0.5 mg of various fractions.

nary mucoprotein (3), Anderson-Maclagan urinary material (8), and Di Ferrante-Popenoe (4) and Maxfield (7) mucosubstance all gave the typical "reaction of identity" with normal R-1 material (Figure 2). CTAB preparations consistently gave a broad zone of precipitin reaction in gels as illustrated in Figure 2. Utilizing lyophilized samples of the above materials, the proportions for optimal antigen-antibody reaction were essentially the same for R-1, Tamm and Horsfall, and Maxfield substances. Approximately 30 per cent more Anderson-Maclagan material was required to give equivalent precipitation.

Rabbit antihuman serum protein antibodies gave no precipitation in tubes or gel with R-1, Tamm and Horsfall or Maxfield substances. A distinct precipitation in tubes and gel was obtained with this antibody and with Anderson-Maclagan material (Figure 3).

The normal RS-1 (nonultrafiltrable, veronal-soluble) urinary preparations gave two distinct zones of precipitation with the anti-R-1 antibody. Fractionation of RS-1 by Cohn method 10 into three primary fractions—RS-1A (Cohn I, II, III), RS-1B (Cohn IV, V), and RS-1C (Cohn VI) (2)—resulted in multiple zones of precipitation with the anti-R-1 antibody and each preparation (Figure 4). When RS-1 material was brought to pH 3 with dilute acetic acid, both the precipitate and supernate gave multiple precipitin zones with the anti-R-1 antibody. CTAB treatment of a solution of RS-1 solids by the method of Maxfield (7) gave multiple zones of precipitation for the supernate and a single line with the precipitate when

tested against anti-R-1 antibody. RS-1 fractions of urine from patients with renal calculi, pyelonephritis, cancer and other inflammatory or degenerative diseases consistently gave precipitin reactions with anti-R-1 antibody (Figure 5).

Rabbit antihuman serum antibody gave multiple precipitation zones with the RS-1 preparations. Absorption of RS-1 with rabbit antihuman serum antibody did not alter the serological response to rabbit anti-R-1 antibody.

The nondialyzable ultrafiltrable solids, UF-O, of urine (13) contained no detectable R-1 reactive substance even at concentrations up to ten times those of optimal concentration for R-1 solids. A precipitate obtained from a solution of UF-O with CTAB failed to give an antigen-antibody precipitation with the anti-R-1 antibody in tubes or gel diffusion.

Presence of R-1 in renal and other tissues. The renal tissue preparations consistently gave a positive precipitin reaction with the rabbit anti-R-1 antibody. The concentration of antigen in the various tissue preparations decreased in the following order: cortex, medulla, pelvis denuded of epithelium, whole pelvis, and epithelium stripped from pelvis. The epithelium required approximately four times the tissue concentration of cortex to give comparable precipitation reactions with the anti-R-1 antibody.

The cortex and medulla commonly gave two zones of precipitation with the antibody; the pelvis and epithelium gave single zones of precipi-

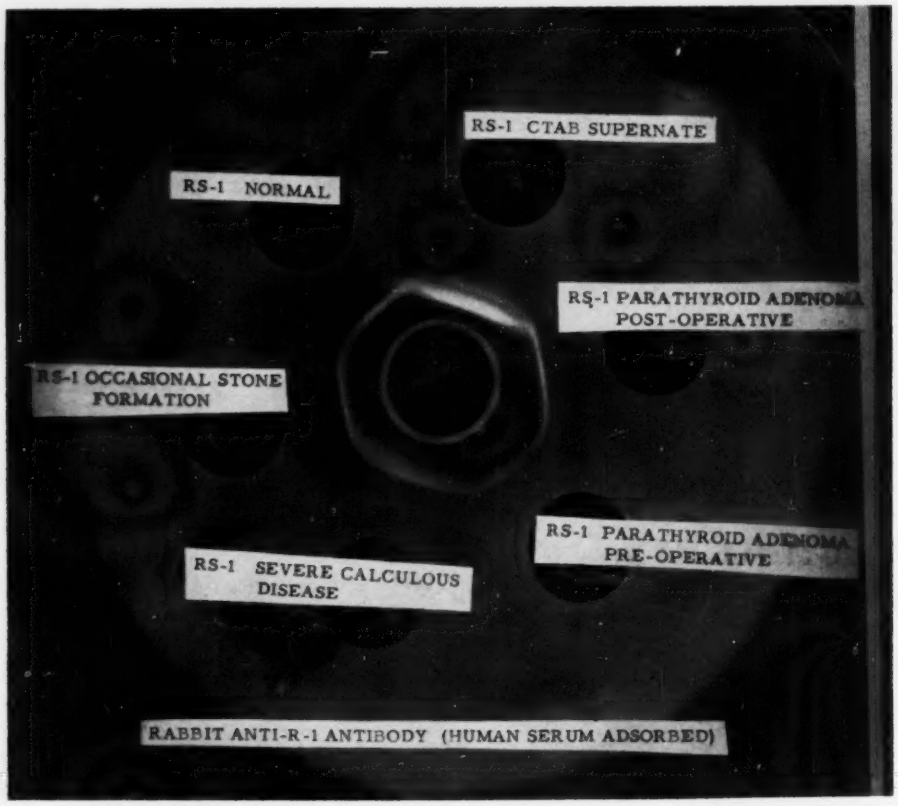

FIG. 5. PRECIPITIN REACTIONS OBTAINED AGAINST ANTI-R-1 ANTIBODY WITH NORMAL RS-1 AND RS-1 URINARY FRACTIONS FROM PATIENTS WITH RENAL CALCULI. Peripheral well 1 contains lyophilized supernate from CTAB treatment of a solution of RS-1. The RS-1 preparations in other peripheral wells are from the same patients as R-1 preparations in Figure 1. Each peripheral well contains approximately 1 mg of test substance.

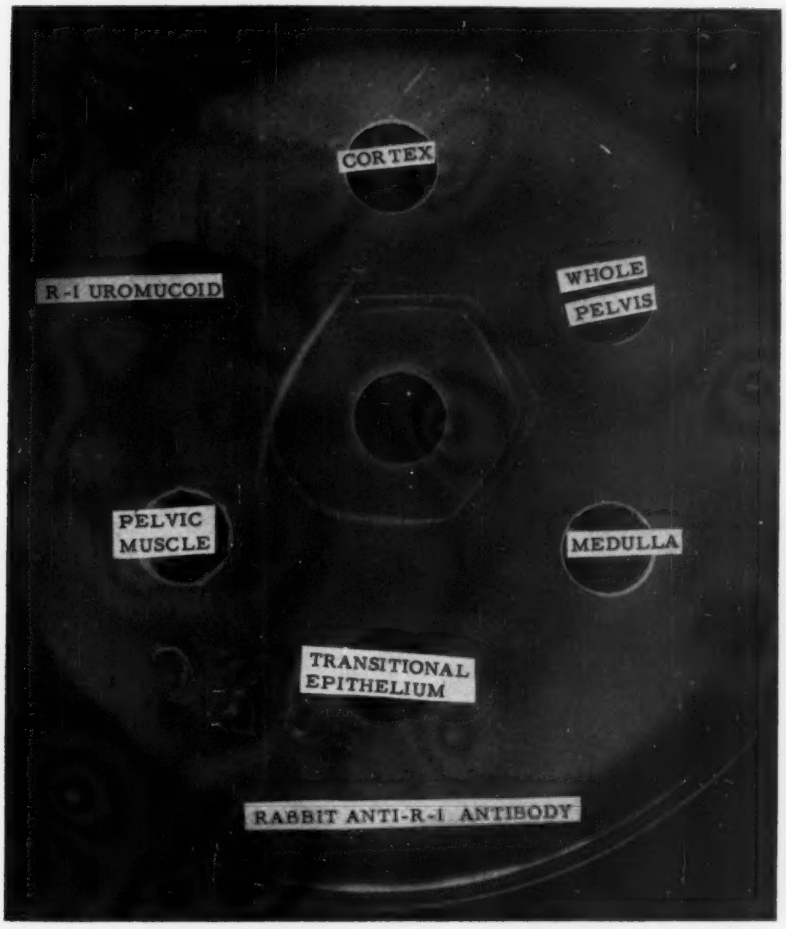

FIG. 6. PRECIPITIN REACTIONS OF URINARY TISSUES AND NORMAL R-1 MUCOID AGAINST ANTI-R-1 ANTIBODY. The tissue preparations contain approximately 300 to 500 mg of suspended solids per well, compared with 1 mg of R-1 substance in well 6. Tube titration of equivalent zone of precipitin reactions of these tissue preparations is not possible because of the opacity of the solutions. Ultracentrifugation to clarity removes all but traces of the anti-R-1 precipitable substance.

tation in gel. Precipitation reactions in gel were obtained from all preparations of skin and subcutaneous fat, lumbodorsal fascia, and striated muscle. These various tissue preparations gave a "reaction of identity" with each other, but the R-1 substance usually gave a distinct spur with the tissue preparations (Figures 6 and 7). Such spur formation has usually been interpreted as a "reaction of partial identity" (12), and persisted through tenfold dilutions of either antibody or antigens. With progressive dilutions of antiserum the spur formation disappeared before complete loss of the tissue precipitin reaction, thus giving the appearance of "reactions of identity" between R-1 and tissue antigens. A similar response was noted in anti-R-1 antibodies of low titer from animals which had not received challenge doses of antigens in 8 months. The spur formation promptly reappeared when the antibody titer was boosted by a challenge dose of R-1 without adjuvant.

Absorption of anti-R-1 antibody with increas-

ing quantities of renal tissue slurry completely absorbed the serological response of the antibody to all of the various tissue slurries. Antiserum thus absorbed continued to give a serological response to urinary R-1 and a single precipitin reaction of identity to urinary RS-1 solids. The less rapidly diffusible of the two zones of precipitin reaction, uniformly present with RS-1 solids diffused against untreated antibody, was absent in all preparations of tissue-absorbed antibody which failed to give a serological response to tissue. It was not possible to remove all serological reactivity of the antibody to R-1 solids by absorption with renal tissue slurries even when the wet weight of tissue (1,000 mg) exceeded the γ-globulin content of the antibody preparation (10 mg) by 100-fold.

On the other hand, the absorption of anti-R-1 antibody with increasing quantities of R-1 resulted in complete loss of serological response of the antiserum to all tissues and to all R-1 and RS-1 preparations. Attempts to absorb the R-1 reactive component of the antiserum by titration with R-1 without absorption of the tissue and RS-1 reactive components were uniformly unsuccessful.

This suggests that R-1 evokes antibody formation to both the largest molecular form of R-1 and to the smaller chain lengths (half or quarter lengths or both). Freshly prepared R-1 precipi-

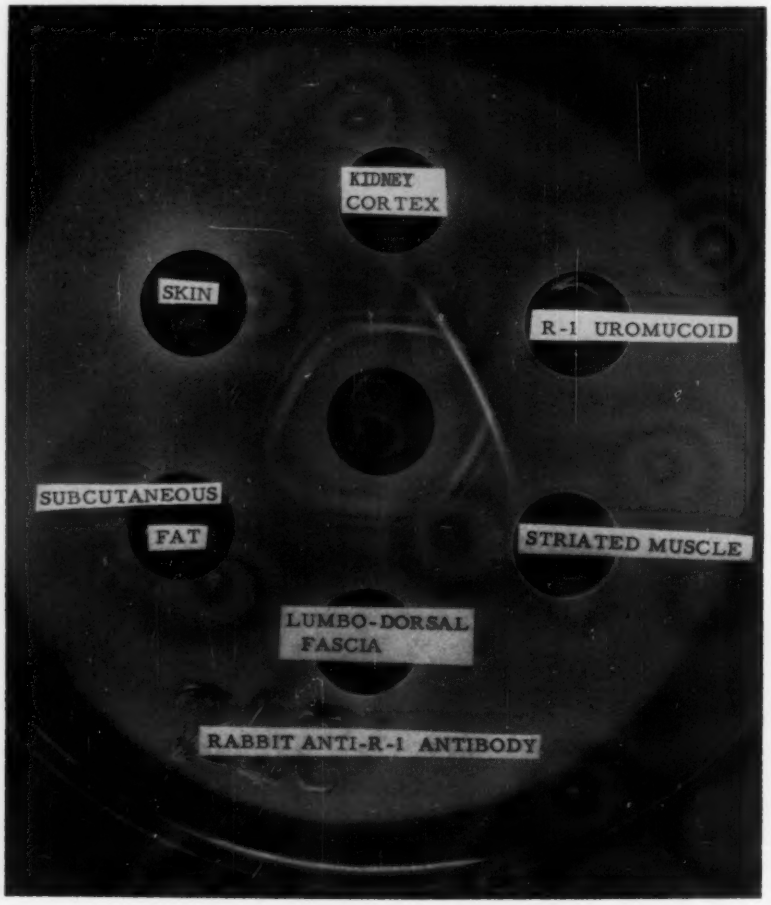

FIG. 7. PRECIPITIN REACTIONS OBTAINED FROM TISSUES OF THE ABDOMINAL WALL AGAINST ANTI-R-1 ANTIBODY.

tates all serologically reactive antibodies of anti-R-1 rabbit serum, although it contains predominantly the longer chain lengths. The tissues contain serologically reactive substance which completely precipitates only the antibodies to short chain lengths of R-1. On the basis of the spur formation between R-1 and tissue serological reactions, the R-1 may be regarded as the "complete antigen," the tissue reactive substance as the "incomplete antigen."

The precipitin reaction of tissue extracts with anti-R-1 serum does not appear to be related to the use of Freund's adjuvant. Similar reactions were obtained with anti-R-1 antibodies produced without adjuvant. The Freund's adjuvant gave no precipitin reaction in tubes or gel with any of the anti-R-1 antibody preparations. Absorption of the anti-R-1 serum with Freund's adjuvant did not alter the precipitin reactions with the tissue extracts.

The various tissue extracts gave multiple zones of precipitation with rabbit antihuman serum antibodies. However, absorption (11) of the tissue extracts with up to five times the optimal concentration of antihuman serum antibody failed to prevent the subsequent response to anti-R-1 antibody. Normal rabbit serum gave no precipitin reaction with any of the tissue extracts, blood plasma, or urinary nondialyzable solids of human origin.

Absence of R-1 in various tissue fluids, bone and stone matrices. The anti-R-1 antibody failed to give any precipitation reaction in tubes or gel with human serum from 12 normal subjects or

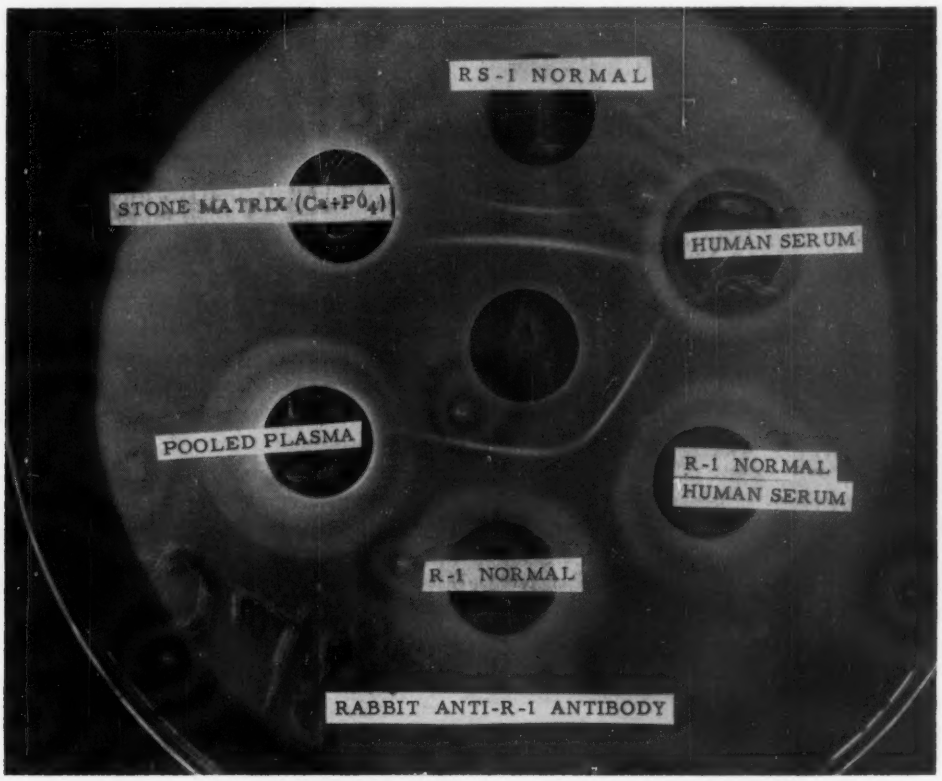

FIG. 8. RECOVERY OF R-1 FROM NORMAL HUMAN SERUM BY ANTI-R-1 ANTIBODY. Well 1 contains 1 mg of RS-1 solids and 0.5 mg of R-1 in 0.4 ml distilled water. Well 2 contains 0.4 ml of fresh normal serum. Well 3 contains 0.5 mg of R-1 in 0.4 ml of normal serum. Well 4 contains 0.5 mg of R-1 in 0.4 ml of distilled water. Well 5 contains 0.4 ml of pooled normal plasma from 10 subjects. Well 6 contains 2 mg of whole freshly prepared renal calcigerous calculus matrix in 0.4 ml of distilled water.

with the serum of the patients from whom the above renal tissue was obtained. Serum from six patients with severe calculous disease and from ten patients with severe debilitating disease, terminal metastatic cancer, hepatic cirrhosis, and recent major surgical procedures, also failed to give precipitation reactions with anti-R-1 antibody. Lyophilized R-1 suspended in fresh normal serum for 24 hours at 3° C or incubated with normal serum at 56° C for 1 hour subsequently gave precipitin reactions with anti-R-1 antibody, and was detectable in dilutions of 1 mg R-1 in 23 ml of plasma (Figure 8). These suspensions of R-1 also gave a "reaction of identity" with untreated R-1 antigen in the gel diffusion plates. Failure of rabbit antihuman serum protein antibodies to give a precipitin reaction with R-1 (Figure 3) is additional evidence that R-1 substance is not present in detectable quantities in human serum.

No evidence of precipitation with anti-R-1 antibody was obtained from the several preparations of erythrocyte membranes, viral hemagglutination inhibitory mucoprotein of human saliva, bone matrix, matrices of urinary calculi (Figure 8), or human seminal plasma. Concentration of these substances was tested over a range of approximately 0.1 to 10 times the optimal concentration of R-1 for the preparations of anti-R-1 antibody.

DISCUSSION

Tamm and Horsfall (3) precipitated mucoprotein(s) from normal urine by addition of NaCl to 0.58 M. The recovery was 25 mg per L, of which 12 mg was soluble in 0.025 M phosphate buffer (pH 6.8) and was found to contain all of the viral hemagglutination inhibitory material. Utilizing a similar technique, Odin (14) recovered 13 mg of viral inhibitory mucoprotein, which contained 8 per cent sialic acid per L of urine. In the earlier reports from this laboratory (1), the substance R-1 was found to occur in quantities approximately eight times that reported by Tamm and Horsfall for viral inhibitory urinary mucoprotein and to contain a quantity of sialic acid varying from 9 to less than 1 per cent, as measured by the diphenylamine reaction. Since Tamm and Horsfall mucoprotein recovered by NaCl precipitation in this laboratory did not lose sialic acid by subsequent dialysis against distilled water or by the ultrafiltration technique, it was assumed that the sialic acid-free portion of R-1 represented a previously undescribed urinary mucoid. This material was demonstrated not to inhibit the hemagglutinating activity of influenza viruses.[1] The term "uromucoid" was therefore applied to this component of the R-1 fraction of normal urine. The demonstration of complete antigenic identity between R-1 uromucoid and Tamm and Horsfall mucoprotein suggests that uromucoid may be Tamm and Horsfall material that has lost its complement of sialic acid. It has been demonstrated (3) that treatment of Tamm and Horsfall mucoprotein with an extract of *Vibrio cholerae* causes loss of inhibitory activity but not of immunological precipitability.

The differences in recovery are apparently accounted for by incomplete precipitation; unlyophilized Tamm and Horsfall material had a solubility in 0.58 M NaCl of 85 mg per L. Since it has been demonstrated that neither lyophilization, dialysis nor ultrafiltration per se will remove sialic acid from the Tamm and Horsfall mucoprotein and that the various preparations of Tamm and Horsfall urinary mucoprotein contain varying amounts of sialic acid, one may assume either that uromucoid has never had 8 per cent sialic acid or that the dialysis of whole urine removes an inhibitor of a neuraminidase which then releases the sialic acid prior to ultrafiltration. Collection and dialysis of urine in the presence of ethylenediamine tetraacetic acid, which should inhibit neuraminidase (15), did not yield an R-1 fraction with a greater sialic acid content than an untreated control. Sialic acid in R-1 was no different in an immediately dialyzed urine as compared with a control which stood 3 days at 4° C before dialysis.

Variation in particle size of Tamm and Horsfall urinary mucoprotein, both in the native state and

[1] Dr. Maurice Hilleman, Merck Institute for Therapeutic Research, reported that neither of two samples submitted, having less than 1 per cent sialic acid, inhibited hemagglutination by Asian influenza virus (Jap. 305–57), Influenza A (Netherlands) or Influenza B (Great Lakes strain). The limited solubility of this material in physiological saline tended to make the results valid for low concentration only.

by mild alterations in physicochemical environment, has been demonstrated. Porter and Tamm (16) examined samples of unlyophilized material by electron microscopy and found the flexible filaments to vary greatly in length. The molecules had a width of about 100 Å, an average length of about 25,000 Å, and a nodose structure with high points spaced at about 110 Å. On the basis of ultracentrifugal and diffusion data, Tamm, Bugher and Horsfall (17) assigned a molecular weight of 7×10^6 as the mean particle size of these filaments. Such relatively mild treatment as heating the mucoprotein at 70° C for 30 minutes in phosphate buffer at pH 6.8 resulted in splitting of the molecule with emergence of a smaller component on ultracentrifugation, a reduction in molecular asymmetry by viscosimetry, and a reduction in electrophoretic mobility.

Maxfield (18) has demonstrated that Tamm and Horsfall mucoprotein of molecular weight 7×10^6 may be separated into units of half-length (mol wt 3.5×10^6) and quarter units (mol wt 1.7×10^6). The half-length units were prepared from Tamm and Horsfall mucoprotein by treatment with CTAB and these units were identical with the virus inhibitory mucoprotein of Di Ferrante and Rich (6). The quarter-length molecules were prepared by prolonged treatment of Tamm and Hosfall mucoprotein with CTAB followed by alcohol. Maxfield suggests that the Tamm and Horsfall material represents the native form of this material in normal urine. The normal urine is presumed to contain an inhibitor which protects the bonds that are broken by alcohol to give the subfractions of Tamm and Horsfall mucoprotein.

The demonstration of broad zones of precipitation in gel when CTAB precipitates of urinary fractions are diffused against anti-R-1 antibody is consistent with the fragmentation of Tamm and Horsfall mucoprotein. The presence of multiple precipitin zones in all Cohn fractions of RS-1 subjected to anti-R-1 antibody is consistent with Maxfield's observation that dialysis of urine permits ethanol to disrupt certain bonds of Tamm and Horsfall mucoprotein and that small quantities of ethanol greatly increase the solubility of these fragments in 0.58 M sodium chloride solutions.

The consistent presence of two zones of precipitation in gel when RS-1 solids are matched with anti-R-1 antibody suggests the presence of more soluble (presumably smaller) components of R-1 in normal urine. The quantity of these may be relatively large since, by planimetric estimate from electrophoretic experiments, the mean concentration of the ten identifiable gradients of RS-1 solids may range between 2 and 19 mg per 24 hours (19). Grant (20) and Vaerman and Heremans (21) found immunoelectrophoretic evidence for at least two forms of Tamm and Horsfall mucoprotein in urine. Grant also noted a "reaction of identity" between urinary mucoproteins precipitated by 0.58 M NaCl, cetyltrimethylammonium bromide and benzoic acid.

The serological response of tissue to anti-R-1 antiserum is probably not due to contamination of R-1 with an antigenically competent molecule of tissue origin. If such a contaminant were present, the serological response of the antibody to tissue should be separable from the response to R-1 by progressive titration of the antiserum with R-1. The tissue contaminant should also have been detectable by ultracentrifugation or electrophoresis of the R-1 preparations.

The demonstration of substances in renal parenchyma, renal pelvis, skin, fat, fascia, and striated muscle which give "reactions of partial identity" with the anti-R-1 antibody suggests the presence of molecules which contain in part the same antigenic determinants as the R-1 molecule. The chemical analysis and ubiquity with which these occur in tissues suggest that they may be components of the "ground substance" in the concept of Gersh and Catchpole (22). The R-1 substance in urine may be derived entirely from the tissues of the urinary system and, since it has been clearly identified only in urine, it may be a secretory product of the renal tubular cells. If this material is transported to the kidneys by the blood plasma, the concentration in plasma is either too small to be detectable by our methods or the antigenically reactive groups are effectively blocked in some manner during the plasma transport. If one accepts the value of 700 ml per minute for renal plasma flow and postulates the complete clearance of R-1 by the kidneys, then a minimal concentration of 1 mg in 11,200 ml of plasma could account for the daily excretion of 90 mg of R-1. Our

methods will not detect plasma concentrations of R-1 below 1 mg in 23 ml.

SUMMARY

The R-1 fraction of normal urine has been demonstrated to be highly antigenic for rabbits, to be capable of producing anaphylaxis, and to retain its antigenic properties under a variety of alterations in physicochemical environment.

Reactions of immunological identity have been established between the R-1 uromucoid and the urinary mucoproteins described by Tamm and Horsfall, Di Ferrante and Popenoe, and by Maxfield. The Anderson and Maclagan benzoic acid-adsorbable material of urine contains both R-1 substance and appreciable quantities of RS-1 solids derived from blood plasma.

Two zones of precipitation occur in gel diffusion reactions between homogenous anti-R-1 antibody and the nonultrafiltrable, veronal-soluble RS-1 components of urine, these zones becoming more numerous after treatment of RS-1 solids with cetyltrimethylammonium bromide, dilute ethanol, or acetic acid to pH 3. This is interpreted as additional evidence that R-1 uromucoid occurs in two or more physical forms in normal urine, the variations being largely a matter of length of the molecule and solubility in various solutions.

An immunological reaction of partial identity has been demonstrated between the human urinary R-1 substance and tissue extracts of human renal parenchyma, renal pelvis, skin, fat, lumbodorsal fascia, and striated muscle from the abdominal wall.

No evidence could be obtained for the presence of substances antigenically reactive to anti-R-1 antibody in the following preparations: matrices from a variety of renal calculi, human blood plasma in health and various disease states, erythrocyte membranes, human saliva, matrix from fetal bone, human seminal plasma, or the nondialyzable ultrafiltrable solids (UF-O) of normal urine.

It is suggested that a substance related to the material found in urinary fraction R-1 may be a component of the "ground substance" of various tissues, but is not a "calcifiable" component of either bone matrix or urinary calculi. The R-1 uromucoid as recovered in urine has not been clearly identified in any body fluid or tissue other than urine.

ACKNOWLEDGMENT

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RENAL ORIGIN OF AN ALDOSTERONE-STIMULATING HORMONE IN DOGS WITH THORACIC CAVAL CONSTRICTION AND IN SODIUM-DEPLETED DOGS

By JAMES O. DAVIS, CARLOS R. AYERS and CHARLES C. J. CARPENTER with the surgical assistance of ALFRED CASPER and the technical assistance of ELEANOR CAVANAUGH

(From the Section on Experimental Cardiovascular Disease, Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, Bethesda, Md.)

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Recent studies (1–5) indicate that the immediate stimulus to aldosterone production is humoral. Evidence for secretion of an aldosterone-stimulating hormone (ASH) by the kidney in response to acute blood loss was presented at the Laurentian Hormone Conference in 1960 (4). The present study is concerned with the locus of secretion of an ASH in two other experimental situations: *1*) hyperaldosteronism secondary to chronic constriction of the thoracic inferior vena cava, and *2*) chronic Na depletion. The present data provide evidence for extension of our knowledge of an ASH to clinical states with edema; the dog with thoracic caval constriction is very similar in regard to electrolyte and water metabolism and hyperaldosteronism to the patient with cirrhosis of the liver and ascites. Studies of the origin of secretion of an ASH during Na depletion were made because changes in electrolyte intake constitute one of the most important physiological mechanisms in the regulation of aldosterone secretion. Evidence for secretion of an ASH by the kidney in these two experimental situations was obtained by study of the effects of acute nephrectomy on aldosterone secretion.

METHODS

In a preliminary study, the acute effects of nephrectomy on aldosterone and corticosterone secretion were observed in 6 dogs with thoracic caval constriction and an intact anterior pituitary. Following control observations, the kidneys were removed and steroid secretion was measured again 1 to 2 hours later; the effects of subsequent intravenous administration of crude saline extracts of each dog's kidneys were studied.

In Experiment I of the definitive study, 10 dogs with chronic caval constriction were subjected to hypophysectomy; the animals were given 75 to 100 mg of cortisone acetate intramuscularly on the day of hypophysectomy and daily for the remainder of the experiment. Two or three days after hypophysectomy the acute effects of bilateral nephrectomy were studied 1 to 2 hours after removal of the kidneys. In 3 of the 10 animals, crude saline extracts of each animal's kidneys were infused intravenously and the effects on steroid secretion observed. Because of a decline in venous pressure after nephrectomy in 5 of the first 6 animals, an additional ligature was placed around the thoracic inferior vena cava in the last 4 dogs and tightened to maintain a constant high venous pressure.

In Experiment II, Na depletion was produced in 8 normal dogs by means of a low Na diet (1 to 2 mEq per day of Na and 26 mEq per day of K) over a period of 10 to 14 days and by administration of 2 ml of Mercuhydrin (meralluride) intramuscularly on at least two occasions. The Na-depleted dogs were hypophysectomized 2 to 3 days before acute nephrectomy was performed and the animals received 75 to 100 mg of cortisone acetate daily as in Experiment I.

All animals were mongrel dogs weighing 15 to 20 kg. The animals were anesthetized lightly with Na pentobarbital for the acute observations. Adrenal vein blood was collected by methods described previously (6); all blood removed was replaced with blood from normal dogs. In some animals an infusion of *l*-norepinephrine was given to sustain adrenal blood flow at a fairly constant rate throughout the experiment. The concentrations of aldosterone and corticosterone in adrenal vein plasma were measured by the double isotope derivative assay of Kliman and Peterson (7). Porter-Silber chromogens were determined by a modification of the procedure of Peterson, Karrer and Guerra (8). The kidney extracts were prepared from each animal's own two kidneys. Two to 3 hours after removal of the kidneys, which remained at room temperature during this time, the 2 kidneys were homogenized in 300 ml of normal saline in a Waring blender for 3 minutes. The homogenate was filtered through cheesecloth and fluid from the pulp of the homogenate was squeezed through the cloth. This filtrate was centrifuged at 8,000 rpm in a Lourdes model AT centrifuge for 10 minutes. The supernatant was infused intravenously at a rate of 5 to 6 ml per minute by means of a constant infusion pump.

RESULTS

In the preliminary studies, aldosterone secretion fell after nephrectomy in only two of the six ani-

TABLE I

Effects of nephrectomy on steroid secretion in dogs with chronic thoracic caval constriction

Dog	Aldosterone secretion* μg/min			Corticosterone secretion μg/min		
	Control	After nephrectomy	After kidney extract	Control	After nephrectomy	After kidney extract
1	0.071	0.012	0.052	2.42	2.19	0.82
2	0.196	0.208	0.183	8.80	7.22	7.97
3	0.061	0.047	0.053	5.60	4.80	3.48
4	0.078	0.086	0.031	3.66	3.88	2.49
5	0.091	0.046	0.148	4.60	3.40	4.91
6	0.138	0.118	0.107	3.64	2.70	2.62
Dogs with thoracic caval constriction stressed by laparotomy	0.135 ±0.056 (N = 16)			3.11 ±1.78 (N = 16)		

* Each value for aldosterone secretion represents the average of 2 to 4 determinations on separate collections of adrenal vein plasma.

mals with thoracic caval constriction and an intact anterior pituitary (Dogs 1 and 5 of Table I). The values for aldosterone secretion during each of the three periods of Table I represent the averages of two to four determinations on separate collections of adrenal vein plasma. Corticosterone secretion was very high during the control period in all but Dog 1 (average value of 4.79 μg per min-

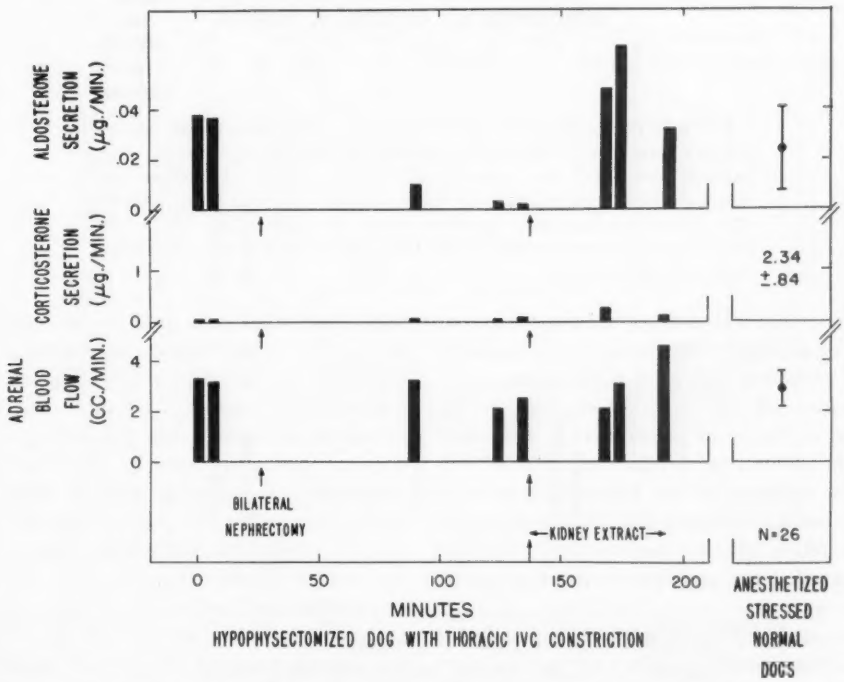

FIG. 1. EFFECTS OF NEPHRECTOMY AND INTRAVENOUS INJECTION OF A KIDNEY EXTRACT ON STEROID SECRETION AND ADRENAL BLOOD FLOW IN A HYPOPHYSECTOMIZED DOG WITH THORACIC INFERIOR VENA CAVAL (IVC) CONSTRICTION. Comparative data for anesthetized stressed normal dogs are presented in the right section.

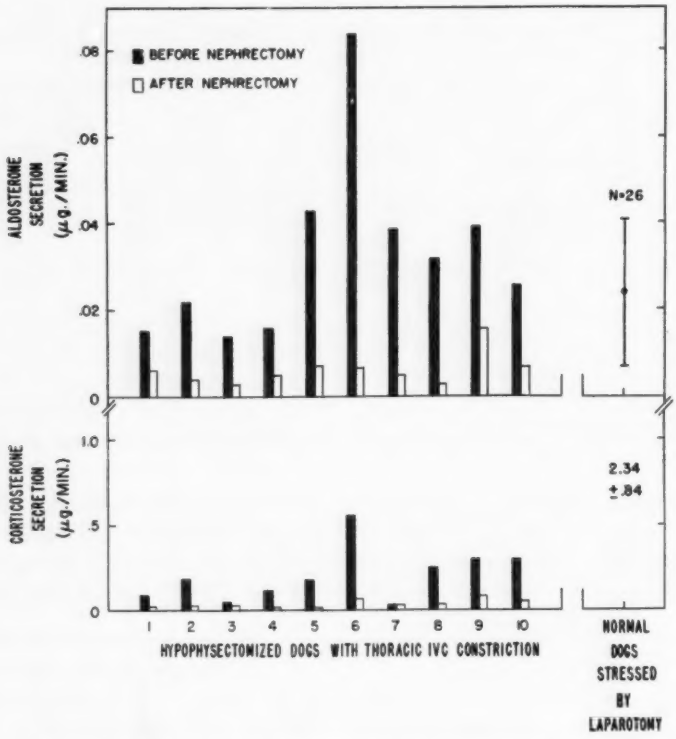

FIG. 2. EFFECTS OF ACUTE NEPHRECTOMY ON ALDOSTERONE AND CORTICOSTERONE SECRETION IN 10 HYPOPHYSECTOMIZED DOGS WITH THORACIC CAVAL CONSTRICTION. Each bar represents the average of 2 or 3 analyses from separate collections of adrenal vein blood. Comparative data from 26 normal dogs stressed by laparotomy are presented on the right. Dogs 4–9 received an infusion of *l*-norepinephrine to maintain arterial pressure and adrenal flow at the control level.

ute in comparison with 3.11 μg per minute for another series of dogs with thoracic caval constriction) and remained high after nephrectomy. Venous pressure fell slightly in all six dogs during the course of the acute experiment. A marked increase in aldosterone production occurred following the infusion of the kidney extract only in the two animals (Dogs 1 and 5) in which aldosterone secretion fell after nephrectomy. Corticosterone secretion was apparently not influenced by the kidney extracts.

Experiment I: Effects of nephrectomy and subsequent administration of kidney extracts on steroid secretion in hypophysectomized dogs with chronic thoracic caval constriction. Because of the high secretion rates for corticosterone and presumably increased ACTH release in the preliminary experiment, the anterior pituitary was removed 2 to 3 days before nephrectomy was performed in the definitive study. The experimental design and the results of a typical experiment are presented in Figure 1 and the findings for all ten dogs are plotted in Figure 2. As a result of hypophysectomy, the average control value of 0.033 μg per minute for aldosterone secretion was considerably lower than the average value of 0.135 μg per minute obtained in dogs with chronic caval constriction and an intact pituitary ($p < 0.01$). However, in several of the animals (Dogs 5–9), the control rates of aldosterone secretion were higher than the average value for normal dogs stressed by laparotomy (right section of Figure 2). After nephrectomy, aldosterone production fell in every animal and, in most instances, the

final rate of aldosterone secretion was very low; this decline in aldosterone secretion was frequently maximal at the end of 1 hour. Aldosterone output decreased from the mean control value of 0.033 μg per minute to an average value after nephrectomy of 0.006 μg per minute ($p < 0.01$), an 82 per cent fall. Although the initial values for corticosterone secretion were low as a result of hypophysectomy, a further decline from 0.20 to 0.04 μg per minute ($p < 0.01$) was demonstrable after nephrectomy. Porter-Silber chromogen output was reduced from an average value of 0.22 to 0.13 μg per minute ($p > 0.05$); although the difference for the group was not statistically significant, the degree of response was sufficiently great in some animals to indicate a definite decrease. Inferior vena caval pressure fell in five of the first six animals. In the last four animals an additional ligature was placed around the thoracic inferior vena cava and tightened during the acute study; venous pressure was maintained at the high control level in all four animals. Adrenal blood flow was unchanged ($p > 0.05$). In three dogs, saline extracts of each animal's two kidneys produced an increase in aldosterone production, but corticosterone and Porter-Silber chromogen secretion increased in only one of the three animals; this animal (Figure 1) showed a 25-fold increase in aldosterone output, which was the greatest response for the group.

Experiment II: Effects of acute bilateral nephrectomy on steroid secretion in Na-depleted hypophysectomized dogs. All eight animals lost weight during the period of low Na intake. After the first injection of meralluride, 70 to 100 mEq of Na was excreted, whereas the second injection resulted in a considerably smaller loss of Na (8 to 40 mEq). The experimental design and the results of a typical acute experiment are presented in Figure 3. The initial control values for aldosterone secretion for these Na-depleted hypophysectomized dogs before nephrectomy were high. The mean control value for the group was 0.054 μg per minute (Figure 4) in comparison with a value of 0.008 μg per minute for simple hypophysectomized dogs (10) ($p < 0.001$) and a value of 0.024 μg per minute for normal dogs stressed by laparotomy ($p < 0.02$).

After nephrectomy, aldosterone secretion fell in every animal; the response was maximal or nearly maximal at the end of the first hour. The average

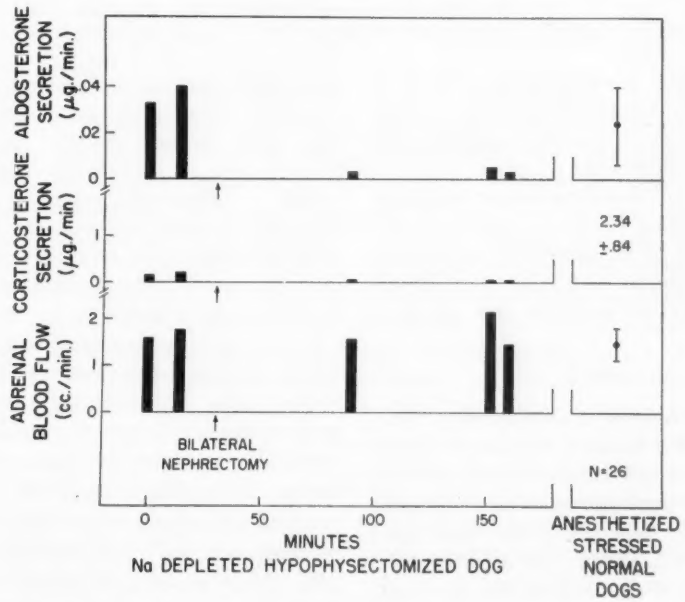

FIG. 3. TYPICAL EFFECTS OF NEPHRECTOMY ON ALDOSTERONE AND CORTICOSTERONE SECRETION AND ADRENAL BLOOD FLOW IN A Na-DEPLETED HYPOPHYSECTOMIZED DOG.

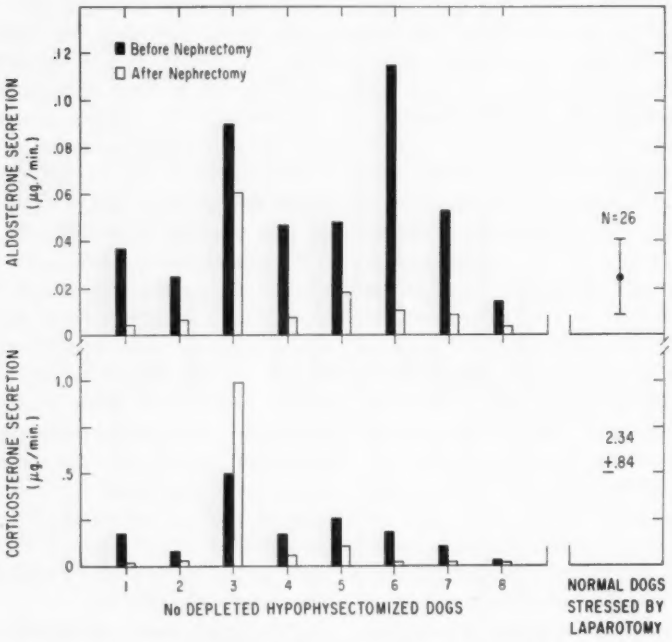

FIG. 4. EFFECTS OF NEPHRECTOMY ON STEROID SECRETION IN 8 Na-DEPLETED HYPOPHYSECTOMIZED DOGS. Each bar represents the average of 2 values from separate collections of adrenal vein blood. Same comparative data as in Figure 2. Only one animal (Dog 3) received an infusion of *l*-norepinephrine to maintain arterial pressure and adrenal blood flow at the control level.

decrease for the group was from 0.054 to 0.015 μg per minute, a 72 per cent fall (Figure 4). In Dog 3, hypophysectomy was apparently incomplete and only a slight fall in aldosterone secretion occurred; a further decrease in aldosterone output was probably blocked by release of ACTH which was reflected by increases in corticosterone and Porter-Silber chromogen output. All other dogs showed a drop in corticosterone secretion after nephrectomy.

DISCUSSION

The immediate stimulus to aldosterone production is mediated via a humoral mechanism (1–5). Indirect evidence for this mechanism was provided by stimulation of hypersecretion of aldosterone by the transplanted and completely denervated adrenal in response to chronic Na depletion (3), chronic thoracic caval constriction (9), and acute blood loss (9). Direct evidence that a humoral agent, an aldosterone-stimulating hormone, promotes increased aldosterone secretion in experimental secondary hyperaldosteronism was obtained by cross circulation of blood from donor dogs with chronic thoracic caval constriction through normal isolated adrenals (1); hypersecretion of aldosterone occurred in the isolated adrenals. Control experiments which involved cross circulation of blood from normal donor dogs through normal isolated adrenals failed to show an increase in aldosterone secretion.

In dogs with thoracic caval constriction and secondary hyperaldosteronism, this aldosterone-stimulating factor is not ACTH. In conscious unstressed dogs with thoracic caval constriction and maximal or near maximal rates of aldosterone secretion, the circulating level of ACTH was at a low basal rate as reflected by extremely low rates of corticosterone and Porter-Silber steroid secretion (5). These experiments clearly demonstrate that ACTH is not the primary humoral factor leading to increased aldosterone production in the dog with caval constriction.

The striking fall in aldosterone secretion which follows hypophysectomy of dogs with thoracic caval constriction and the complete blocking effects of this response by ACTH, indicate that ACTH is another important humoral agent in the control of aldosterone secretion. The marked effects of hypophysectomy have been observed within 1 to 2 hours after pituitary ablation in anesthetized dogs with caval constriction and throughout the entire post-hypophysectomy period of 1 week in conscious dogs with caval constriction (5). However, as indicated previously, it is clear that only a basal output of ACTH is required for maximal aldosterone secretion; an 8- to 30-fold increase in aldosterone secretion occurred following thoracic caval constriction in conscious dogs, with no appreciable increase in corticosterone or Porter-Silber steroid secretion (5). These findings have led to the suggestion that ACTH supports the biogenesis of aldosterone at a very high level, whereas ASH provides the immediate stimulus to aldosterone secretion in dogs with thoracic caval constriction (4).

In a previous study (10), it was demonstrated that an ASH is secreted by the kidney in response to acute blood loss. Also, extensive ablation experiments indicated that this aldosterone-stimulating factor is not secreted by the anterior pituitary, the pineal gland, the brain or the liver. The present data provide evidence for the renal origin of an ASH in response to chronic thoracic caval constriction and to chronic Na depletion. Removal of the kidney from hypophysectomized dogs with thoracic caval constriction resulted consistently in a marked drop in aldosterone secretion. Failure of aldosterone secretion to fall after nephrectomy in four of the six dogs in which the anterior pituitary was present, probably reflects the large amount of ACTH release secondary to the stress of laparotomy for adrenolumbar vein cannulation; corticosterone secretion, which reflects the level of circulating ACTH, was very high. That circulating ACTH was effecting essentially a maximal rate of aldosterone production in these four dogs is also indicated by failure of aldosterone secretion to increase during infusion of the kidney extracts, whereas a striking response to the kidney extracts occurred in the other two dogs in which aldosterone production fell after nephrectomy. Similar results were obtained by Denton, Goding and Wright (3) after nephrectomy in sheep with the anterior pituitary intact. In one of the present Na-depleted animals (Dog 3, Figure 4) in which hypophysectomy was incomplete, the data clearly indicate that the occurrence of a slight reduction in aldosterone secretion rather than the usual marked fall was due to ACTH release; corticosterone production and Porter-Silber chromogen secretion were elevated after removal of the kidneys. In the definitive study in which the anterior pituitary was removed before nephrectomy of dogs with caval constriction, the drop in both aldosterone and corticosterone secretion was consistent and marked. Indeed, the 82 per cent fall in the secretion of aldosterone is the same degree of response observed following hypophysectomy of dogs with thoracic caval constriction (11). In the Na-depleted animals in which hypophysectomy was complete, a similar marked drop in both aldosterone and corticosterone output occurred after nephrectomy. It appears that loss of ACTH and subsequently of ASH leaves the adrenal cortex with a very low rate of aldosterone and corticosterone production which is dependent on a basic intrinsic mechanism of steroidogenesis.

No control experiments on sham-operated animals were performed. This seemed unnecessary since acute nephrectomy was performed on animals which were laparotomized 1 to 2 hours earlier for adrenal vein cannulation and no additional surgery was required for nephrectomy except ligation of the renal vessels and extirpation of the kidneys. It appears unlikely that the metabolic changes which occur after nephrectomy could have influenced aldosterone secretion appreciably, since the fall in aldosterone secretion was maximal or near maximal within 1 hour after removal of the kidneys.

The initial control values for aldosterone secretion in both the hypophysectomized caval and the hypophysectomized Na-depleted dogs were consistently greater than the rates of aldosterone secretion observed in simple hypophysectomized dogs and frequently higher than aldosterone secretion in normal dogs stressed by laparotomy. This finding indicates that hypersecretion of aldosterone occurred in the absence of the anterior pituitary both in the Na-depleted animals and in the dogs with caval constriction. This continued

hypersecretion of aldosterone in the absence of anterior pituitary hormones is presumably secondary to increased release of ASH. The data are in agreement with earlier evidence (5) that increased secretion of aldosterone occurs in response to caval constriction in the conscious hypophysectomized dog. The high initial values for aldosterone secretion in some of the Na-depleted hypophysectomized dogs indicate that chronic Na-depletion in the dog is a much more effective stimulus to aldosterone production than had heretofore been found; Rosnagle and Farrell (12) observed only a twofold elevation in Na-depleted dogs with the pituitary present.

The present experimental conditions and results are in contrast to an earlier experiment (11) in which normal dogs were hypophysectomized and subjected to a low Na diet for 7 to 19 days but were not given meralluride to reduce total body Na. In this earlier study, aldosterone secretion was apparently increased in some of these simple hypophysectomized dogs by Na depletion but hypersecretion was not achieved. In the present experiments, Na depletion was carried out in normal dogs for 10 to 14 days, which was probably adequate time for hypertrophy of the zona glomerulosa to occur, and measurements of aldosterone secretion were made 2 to 3 days after hypophysectomy in Na-depleted dogs supported with cortisone to maintain cardiovascular function and adrenal blood flow; the response in aldosterone production to Na depletion was evident after hypophysectomy. Failure of hypersecretion of aldosterone to occur after acute constriction of the thoracic inferior vena cava in anesthetized hypophysectomized dogs (11) is probably attributable to the inability of the animals to withstand the marked acute alterations in cardiovascular function in the absence of anterior pituitary hormones. It is frequently necessary to support the anesthetized hypophysectomized dog with *l*-norepinephrine or cortisone; the latter was used consistently in the present study to support cardiovascular function. The rates of aldosterone secretion in the present cortisone-treated hypophysectomized dogs with caval constriction are higher than those observed previously in such animals within 1 to 2 hours after hypophysectomy. It appears that the sudden loss of the anterior pituitary hormones has a marked influence on steroid secretion and that compensation is partially achieved within 2 to 3 days. This increase in aldosterone production during the first 2 to 3 days after hypophysectomy of dogs with caval constriction has been reported previously (5), and rates of aldosterone production from six to ten times normal were achieved.

The finding that an ASH is secreted by the kidney in three different experimental conditions may indicate the presence of an ASH common to all three situations. Secretion of an ASH by the kidney during Na depletion suggests that ASH is an important physiological regulator of aldosterone secretion since alterations in electrolyte intake constitute one of the most important mechanisms in the normal daily regulation of aldosterone secretion.

The crude kidney extracts from one of the three hypophysectomized dogs with caval constriction increased corticosterone and Porter-Silber chromogen secretion; this result occurred in the animal with the greatest increase in aldosterone production (a 25-fold increase). Also, in another hypophysectomized dog with caval constriction, a crude kidney extract increased aldosterone secretion tenfold and both corticosterone production and Porter-Silber chromogen output were augmented (personal observations). In contrast, kidney extracts from normal material increased aldosterone secretion consistently but failed to augment corticosterone and Porter-Silber chromogen output (10). These results may reflect a greater amount of the active agent in the kidneys of dogs with caval constriction than that present in normal dog kidneys. This aldosterone-stimulating factor is clearly not ACTH which is bound to the kidney (13), since the pattern of steroid response of the kidney extracts is distinctly different from that of ACTH. The primary effect of the kidney extracts was on aldosterone production, whereas ACTH produced a greater response in cortisol and corticosterone secretion than in aldosterone output. It is of interest in this connection that "renin"[1] and angiotensin (hypertensin II) effected a marked increase in aldosterone and corticosterone production in the nephrectomized, hypo-

[1] "Renin" has been placed in quotation marks to emphasize the lack of purity of preparations referred to here and cited throughout the literature as renin.

physectomized dog, whereas the Porter-Silber chromogen response was relatively less (14 and personal observations). Although crude extracts from normal kidneys have failed to show corticosterone- and cortisol-stimulating activity (10), fractionation of such extracts has consistently yielded aldosterone-, corticosterone- and cortisol-stimulating activity in the "renin" fractions which were precipitated with 1.7 and 2.5 M ammonium sulfate (15); pressor activity was always associated with aldosterone-stimulating activity. This finding, the decreases in aldosterone, corticosterone and Porter-Silber chromogen output after nephrectomy of dogs with caval constriction, and the effects of 50 per cent of the kidney extracts from these animals on the secretion of all three steroids, are consistent with the identity of ASH and "renin" or a renin-like substance. It appears that ASH has a biosynthetic action either at an early stage of the steroidogenic process or on multiple loci in biosynthesis, since not only aldosterone but corticosterone and cortisol synthesis are influenced.

What is the stimulus to ASH release and where are the effector cells located in the kidney? After acute blood loss, it is easy to visualize an acute decrease in pressure or volume leading to ASH release. Also, during chronic Na depletion the same stimulus could be operative. A plausible location for the effector cells is suggested by hypergranulation of the juxtaglomerular cells during Na depletion (16). Also, recent studies (17) have demonstrated hypergranulation and occasional hyperplasia of the juxtaglomular cells in dogs with hyperaldosteronism secondary to thoracic caval constriction. Since the juxtaglomerular cells are located in the media of the renal afferent arterioles, the possibility must be strongly considered that the afferent arterioles constitute the receptor site.

SUMMARY AND CONCLUSIONS

The effects of nephrectomy on aldosterone and corticosterone secretion have been studied in 16 dogs with chronic thoracic caval constriction and in 8 chronic Na-depleted animals. In the preliminary study with the anterior pituitary intact, aldosterone secretion failed to decrease consistently after nephrectomy, apparently because of the high circulating level of ACTH. Consequently, for the definitive study, hypophysectomy was performed before nephrectomy. In the hypophysectomized dogs with thoracic caval constriction, nephrectomy resulted in a fall in aldosterone secretion in every animal from an average control value of 0.033 to 0.006 μg per minute, an 82 per cent fall, and corticosterone output decreased from a very low rate of secretion of 0.20 to 0.04 μg per minute, an 80 per cent fall. A similar striking drop in both aldosterone and corticosterone secretion followed nephrectomy in the Na-depleted hypophysectomized dogs. The high initial control values for aldosterone secretion before nephrectomy of both hypophysectomized caval and hypophysectomized Na-depleted dogs demonstrated hypersecretion of aldosterone in the absence of the anterior pituitary. The findings provide evidence for increased secretion of an aldosterone-stimulating hormone by the kidney in response to chronic thoracic caval constriction and to chronic Na depletion.

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EFFECT OF ORGANOMERCURIALS ON THE RENAL CONCENTRATING OPERATION IN HYDROPENIC MAN: COMMENTS ON SITE OF ACTION*

By JEROME G. PORUSH,† MARVIN H. GOLDSTEIN,† GILBERT M. EISNER† AND MARVIN F. LEVITT

(From the Section of Renal Diseases, Department of Medicine, The Mount Sinai Hospital, New York, N. Y.)

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The renal site of action of organomercurials has variously been placed within the proximal or distal tubule on the basis of numerous observations in dog and man (1–9). Experiments in maximally hydrated man recently reported from this laboratory showed that free water clearance remained relatively fixed throughout the major portion of the diuresis induced by the organomercurial, meralluride (10). In addition, free water clearance could generally be augmented by superimposing a nonspecific solute diuretic [1] during the mercurial diuresis. A hypothesis was suggested that placed a major inhibitory action of organomercurials at a late distal segment, beyond the water-clearing site where, it was assumed, a process of isosmotic salt and water reabsorption takes place.

Previous data concerning the effect of mercurial diuresis on the concentrating operation in hydropenic man have led to varied conclusions. Some have argued that a mercurial diuresis limits the renal concentrating capacity (11). Others have found that a mercurial diuresis superimposed upon or developing simultaneously with a nonspecific solute diuresis does not limit the capacity of the collecting duct to extract solute-free water (12, 13).

Medullary hypertonicity is established primarily by the deposition within the interstitium of salt absorbed from the ascending limb of the loop of Henle. Under conditions of maximal hydropenia, the variable hypotonicity produced in the ascending limb is dissipated by the outward diffusion of water through a permeable tubular membrane, so that isotonicity is reattained before the end of the distal tubule (14–16). The quantity of solute-free water (T^cH_2O) extracted from this isosmotic fluid as it courses through the collecting duct may afford some measure of the quantity of solute transported to the medullary interstitium.

It appeared reasonable that a comparison of the urinary concentrating characteristics during a mercurial diuresis and a nonspecific solute diuresis might elucidate further the site of action of organomercurials in man.

MATERIALS AND METHODS

Four separate groups of studies were performed in normal, maximally hydropenic subjects. In the first or control group, the characteristics of the urine were studied during the infusion of hypertonic mannitol or salt and after the administration of aminophylline. In the second series of experiments, after different rates of solute excretion had been established and maintained at a steady state, an organomercurial was administered intravenously. In the next group of studies, one of the nonspecific solute diuretics used in the control group was superimposed after a mercurial diuresis had developed. Finally, glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were reduced during the course of comparable diureses produced by the infusion of hypertonic salt or the administration of an organomercurial.

All subjects were normal young females, free of cardiovascular or renal diseases. Each was maintained on a regular diet but was deprived of food and water for 16 hours prior to the study, which began at 8:00 a.m. Twelve hours before the experiment, each subject received an intramuscular injection of 2.5 U of vasopressin (Pitressin tannate in oil). Throughout each experiment the infusion contained sufficient aqueous vasopressin to provide at least 200 mU per hour. When filtration rate or renal plasma flow or both were measured, quantities of inulin and para-aminohippuric acid sufficient to produce satisfactory plasma levels were included in the infusion following a priming dose of each agent. All subjects were recumbent during the experiments. Catheterization was performed with a no. 16 French catheter,

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† United States Public Health Service Postdoctoral Research Fellow, National Heart Institute, Bethesda, Md.

[1] The term "nonspecific solute diuretic" is used for those agents which act to increase the quantity of isosmotic fluid escaping proximal tubular reabsorption.

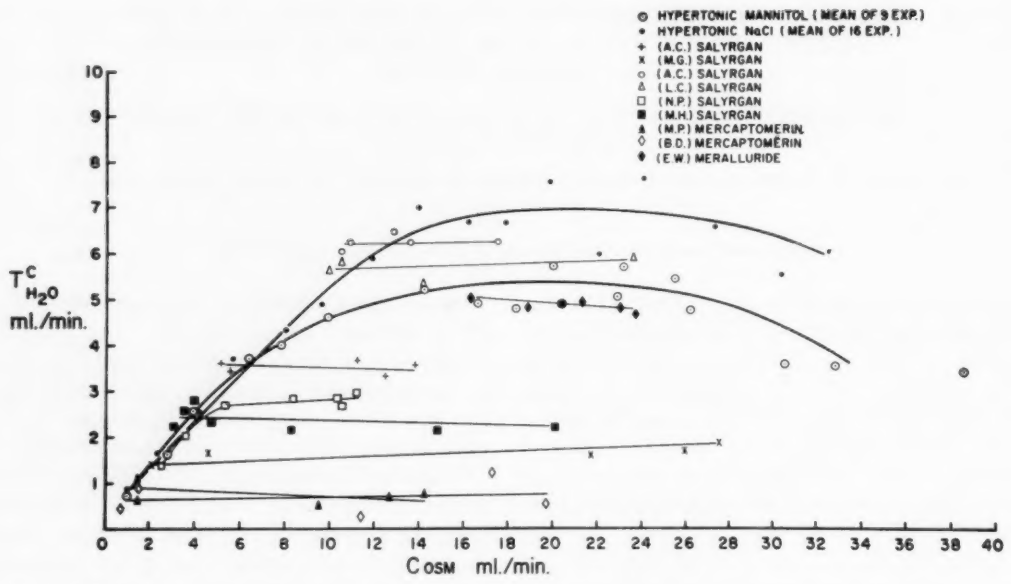

FIG. 1. EFFECT OF ORGANOMERCURIALS AND HYPERTONIC SALT AND MANNITOL INFUSIONS ON SOLUTE EXCRETION (C_{Osm}) AND FREE WATER REABSORPTION (T^cH_2O). In the mercurial experiments the first point represents the value obtained after the fall in T^cH_2O and C_{Osm} before diuresis which was noted in some experiments with Salyrgan and mercaptomerin, and after the first phase of the meralluride diuresis.

and air washouts were used to assure complete bladder emptying. A urine specimen was collected at the beginning of each experiment and at approximately 10 to 30 minute intervals thereafter, depending upon the rate of urine flow. A blood specimen was taken prior to the start of the infusion and every 45 to 60 minutes thereafter from an antecubital vein through a heparinized indwelling needle. The infusions were maintained at a constant rate by a Bowman infusion pump.

In the first group of studies, 9 subjects received an infusion of 10 per cent mannitol, administered at progressively increasing rates from 10 to 35 ml per minute. In a similar manner, 2.5 per cent salt was infused into 16 subjects. When possible, the experiments were continued until a urine flow of 25 to 40 ml per minute had been reached. In 4 subjects, 500 mg of aminophylline was administered intravenously over a 5-minute period to comparably hydropenic subjects.

In the second series of experiments, 26 similarly hydropenic subjects were infused with either normal saline at 1.5 ml per minute or 10 per cent mannitol at 1.5 or 4.5 ml per minute. After a steady state was achieved, three 20-minute urine samples were obtained. Thereafter, 3 ml of meralluride, mercaptomerin, or mersalyl (Salyrgan without theophylline) was injected intravenously. After administration of the mercurial, each experiment was continued for approximately 200 minutes.

In the third group of experiments, approximately 100 to 120 minutes after an organomercurial was administered and a considerable diuresis established, 12 subjects received an infusion of 10 per cent mannitol, 2.5 per cent salt or 500 mg of aminophylline, as in the control experiments. Thereafter the experiments were continued for another 60 to 90 minutes.

In the fourth group of experiments, the blood pressure was suddenly reduced during a Salyrgan- or meralluride-induced diuresis in 4 subjects, and during a diuresis produced by the infusion of 3 per cent salt at 6.5 ml per minute in 3 subjects. This fall in blood pressure was accomplished by the intravenous administration of 50 to 100 mg of SC1950 (1-ethyl-2,6-dimethylluptidine ethobromide),[2] a ganglionic blocking agent, after the application of venous tourniquets to the thighs. The hypotension was maintained for 30 to 60 minutes. Thereafter the blood pressure returned to control levels spontaneously or after the removal of the tourniquets.

All urine and plasma samples were analyzed for osmolality, sodium, potassium and chloride concentrations. When appropriate, inulin and para-aminohippuric acid determinations were also carried out. All determinations were done by methods previously described from this laboratory (17). Clearances were calculated by standard methods.

Solute clearance (C_{Osm}) was calculated from the formula $C_{Osm} = U_{Osm}\ V/P_{Osm}$, where U_{Osm} represents urine osmolality, V is urine flow and P_{Osm} is plasma osmolality.

[2] This drug was generously supplied by G. D. Searle Co., Chicago, Ill., through Dr. Irwin C. Winter, Clinical Director.

TABLE I

Summary of effect of organomercurials on solute excretion and free water reabsorption in hydropenic man

Subject	Mercurial		Solute excretion* (UV/P)	Free water reabsorption (T^cH_2O)
			ml/min	*ml/min*
N.P.	Salyrgan	I	2.2	1.3
		Ia	5.2	2.7
		II	11.1	2.9
M.G.	Salyrgan	I	4.6	1.6
		II	27.8	1.8
E.P.	Salyrgan	I	3.0	1.6
		II	9.2	2.0
H.B.	Salyrgan	I	2.5	1.6
		Ia	4.8	3.1
		II	10.3	2.5
M.H.	Salyrgan	I	3.7	2.6
		II	20.1	2.1
L.J.	Salyrgan	I	4.0	2.6
		II	23.7	2.9
A.C.	Salyrgan	I	4.3	3.0
		II	13.8	3.5
L.G.	Salyrgan	I	5.5	3.6
		II	17.4	3.3
A.S.	Salyrgan	I	8.3	4.7
		II	20.0	4.1
L.C.	Salyrgan	I	10.4	5.8
		II	23.6	5.9
A.C.	Salyrgan	I	10.8	6.2
		II	17.4	6.2
B.D.	Mercaptomerin	I	1.3	0.9
		II	19.7	0.5
M.P.	Mercaptomerin	I	1.4	0.6
		II	12.6	0.7
J.R.	Mercaptomerin	I	1.6	1.1
		II	11.0	0.8
M.R.	Mercaptomerin	I	3.4	2.4
		II	11.4	2.2
E.P.	Mercaptomerin	I	5.1	3.3
		II	16.0	4.0
T.C.	Mercaptomerin	I	9.7	4.2
		II	24.0	4.6
Q.R.	Mercaptomerin	I	12.5	7.0
		II	18.3	8.0
A.S.	Meralluride	I	2.9	1.8
		Ia	6.7	3.0
		II	10.5	3.4
H.L.	Meralluride	I	3.3	1.9
		II	9.0	1.8
R.M.	Meralluride	I	4.1	2.5
		Ia	7.5	3.6
		II	13.6	3.3
J.P.	Meralluride	I	6.2	3.4
		II	13.6	3.6
M.P.	Meralluride	I	5.5	3.5
		II	15.3	4.0
C.D.	Meralluride	I	5.8	4.0
		II	12.5	3.2
E.W.	Meralluride	I	16.2	5.0
		II	23.9	4.6
P.M.	Meralluride	I	13.3	6.9
		II	20.6	6.5

* I represents the period prior to the onset of the mercurial diuresis (after early transient diuresis with meralluride and after initial decrease in C_{Osm} and T^cH_2O in ten of the Salyrgan and mercaptomerin experiments); Ia represents the period showing the small increase in T^cH_2O with the initial rise in C_{Osm} during the mercurial diuresis; II represents the period of peak mercurial effect.

When the urine is concentrated above isotonicity, C_{Osm} is greater than V. Thus, the amount of solute-free water (T^cH_2O) absorbed from isosmotic fluid to concentrate the urine is calculated from the formula $T^cH_2O = C_{Osm} - V$.

RESULTS

Nonspecific solute diuretics (*Figure 1*). In these control studies, all C_{Osm} and corresponding T^cH_2O values obtained for each nonspecific diuretic were grouped about the closest successive 2-ml increment in C_{Osm}. The data represent the mean values for each group.

During hypertonic mannitol infusions,[3] as C_{Osm} increased from 1 to 14.0 ml per minute, there occurred a progressive increase in T^cH_2O from 0.7 to 5.4 ml per minute. Between a C_{Osm} of 14.0 and 23.1 ml per minute, T^cH_2O remained essentially unchanged and thereafter as C_{Osm} increased to 38.6 ml per minute, T^cH_2O declined to 3.6 ml per minute. With hypertonic salt qualitatively similar results were obtained, so that as C_{Osm} increased from 1.3 to 13.7 ml per minute, T^cH_2O increased

[3] Similar results were obtained with isotonic mannitol infusions.

from 0.9 to 6.9 ml per minute. Between a C_{Osm} of 13.7 and 27.3 ml per minute, T^cH_2O remained relatively fixed, and then as C_{Osm} was further increased to 32.6 ml per minute, T^cH_2O tended to diminish slightly to 6.1 ml per minute. After the administration of aminophylline, as C_{Osm} increased from 2.0 to 12.4 ml per minute, T^cH_2O rose from 1.4 to 6.6 ml per minute, producing a curve very similar to that observed after the administration of hypertonic salt.

Organomercurials (Tables I and II, Figure 1). The major diuresis induced by organomercurials began approximately 70 minutes after intravenous administration. The peak effect occurred at 140 minutes and a considerable diuresis was still present after 3 hours.

In 10 of 18 experiments the administration of Salyrgan or mercaptomerin caused a slight fall in C_{Osm} and T^cH_2O, averaging 0.9 ml per minute for each, prior to the onset of the diuresis (Table II; M.G., L.C.). In 4 experiments (Tables I and III, N.P., H.B., A.S., R.M.; Figure 1, N.P.), at the onset of the major mercurial diuresis, an increase in T^cH_2O averaging 1.3 ml per minute (range, 1.1 to 1.5) was observed in association with an initial mean increase in urine flow of 1.8 ml per minute (range, 0.8 to 2.6) and C_{Osm} of 3.1 ml per minute (range, 2.3 to 3.8).

In each meralluride experiment an immediate but transient increase in C_{Osm} averaging 4.3 ml per minute (range, 2.7 to 6.5) was associated with a mean increase in T^cH_2O of 2.2 ml per minute (range, 1.5 to 3.2) (Table II, J.P.; Figure 2).

TABLE II

The effect of organomercurials on solute excretion and free water reabsorption in hydropenic man (three typical experiments)

Subject	Time	Urine flow (V)	Urine osmolality (U)	Solute clearance (UV/P)	Free water reabsorption (T^cH_2O)	Inulin clearance (UV/P)
	min	*ml/min*	*mOsm/kg*	*ml/min*	*ml/min*	*ml/min*
M.G.	Control	1.3	801	3.8	2.5	110
		Salyrgan (3 ml)				
	0 – 29.5	0.7	813	2.1	1.4	72
	29.5 – 58	3.0	430	4.6	1.6	93
	58 – 74	20.1	301	21.7	1.6	125
	74 – 82.5	26.0	298	27.8	1.8	115
	82.5 – 89	24.3	300	26.0	1.7	98
J.P.	Control	1.1	730	2.9	1.8	112
		Meralluride (3 ml)				
	0 – 12.5	4.1	580	8.9	4.8	160
	12.5 – 25	3.3	662	8.0	4.7	
	25 – 42	3.4	687	8.6	4.8	
	42 – 55.5	2.7	677	6.9	4.2	
	55.5 – 65.5	3.2	653	7.9	4.7	
	65.5 – 75	3.3	628	7.8	4.5	131
	75 – 89	2.8	590	6.2	3.4	100
	89 – 100.5	3.4	532	6.9	3.5	
	100.5 – 111	4.9	463	8.5	3.6	
	111 – 122	6.7	420	10.6	3.9	
	122 – 135.5	8.3	383	11.9	3.6	
	135.5 – 145.5	9.0	365	12.3	3.3	104
	145.5 – 155	10.0	359	13.6	3.6	111
	155 – 164	9.9	362	13.5	3.6	
	164 – 173.5	9.9	362	13.5	3.6	
L.C.	Control	4.6	668	11.5	6.9	111
		Salyrgan (3 ml)				
	0 – 11.5	4.3	622	9.9	5.6	83
	11.5 – 31.5	4.6	610	10.4	5.8	80
	31.5 – 56	4.9	559	10.5	5.1	69
	56 – 81.5	8.8	439	14.1	5.3	81
	81.5 – 99	17.7	364	23.6	5.9	114

TABLE III

The effect of nonspecific solute diuretics superimposed on organomercurials on solute excretion and free water reabsorption (two typical experiments)

Subject	Time	Urine flow (V)	Urine osmolality (U)	Solute clearance (UV/P)	Free water reabsorption (T^cH_2O)	Inulin clearance (UV/P)
	min	*ml/min*	*mOsm/kg*	*ml/min*	*ml/min*	*ml/min*
E.P.	Control	2.0	861	5.8	3.8	95
		Mercaptomerin (3 ml)				
	0 – 34.5	2.0	842	5.9	3.9	102
	34.5 – 51	1.8	801	5.1	3.3	95
	51 – 68.5	5.0	553	8.5	3.5	
	68.5 – 79.5	6.6	446	10.3	3.7	90
	79.5 – 91.5	9.5	395	13.2	3.7	96
	91.5 – 102	10.5	377	14.0	3.5	92
	102 – 114.5	11.5	372	15.2	3.7	94
	114.5 – 126.5	12.0	375	16.0	4.0	96
		Aminophylline (500 mg i.v.)				
	126.5 – 137.5	17.5	361	22.6	5.1	117
	137.5 – 149	16.2	387	22.4	6.2	117
N.P.	Control	1.0	657	2.4	1.4	122
		Salyrgan (3 ml)				
	0 – 15	0.9	657	2.2	1.3	105
	15 – 37	0.8	682	2.1	1.3	62
	37 – 61.5	1.5	634	3.6	2.1	62
	61.5 – 83.5	2.5	528	5.2	2.7	
	83.5 – 110	5.5	401	8.3	2.8	
	110 – 128	7.4	366	10.2	2.8	62
	128 – 139	7.8	355	10.4	2.6	63
	139 – 147.5	8.2	355	11.1	2.9	69
		Mannitol (10%)				
	147.5 – 164	12.5	311	15.7	3.2	68
	164 – 175.5	21.8	315	25.5	3.7	76
	175.5 – 189	25.9	315	30.0	4.1	77
	189 – 200.5	32.2	326	38.1	5.9	104

Thereafter C_{Osm} and T^cH_2O returned toward control levels within 30 to 60 minutes.

After the minor variations in T^cH_2O occurring before the major solute diuresis, the characteristics of all 26 mercurial diureses were similar. Specifically, C_{Osm} rose an average of 10.1 ml per minute (range, 3.8 to 23.2) with no mean change in T^cH_2O. T^cH_2O increased a mean of 0.4 ml per minute (range, 0.1 to 1.0) in 13, decreased a mean of 0.4 ml per minute (range, 0.1 to 0.8) in 12, and did not change in 1 subject. This constancy of T^cH_2O obtained despite the marked variation in the levels of C_{Osm} (range, 1.3 to 16.2 ml per minute) and T^cH_2O (range, 0.9 to 7.0 ml per minute) that existed prior to the onset of the major mercurial diuresis. There were no measurable differences among the three mercurials in their effect on T^cH_2O despite a mean increase in C_{Osm} after Salyrgan of 11.8 ml per minute, after mercaptomerin of 11.3 ml per minute and after meralluride of 6.8 ml per minute.

The glomerular filtration rate was determined in 15 of 18 experiments with Salyrgan and mercaptomerin. In 14, a mean decrease in GFR of 26 per cent (range, 13 to 45 per cent) occurred within 40 minutes of administration, prior to the onset of the solute diuresis. This was associated with a fall in C_{Osm} and T^cH_2O in 10 cases, as noted above (Table II, M.G., L.C.). In 8, the GFR slowly returned toward control levels and in 6 the decrease in GFR persisted throughout the experiment. In 6 meralluride experiments in which glomerular filtration rate was measured, an immediate increase occurred, averaging 39 per cent (range, 11 to 64 per cent) (Table II, J.P.). This rise generally disappeared within 50 minutes.

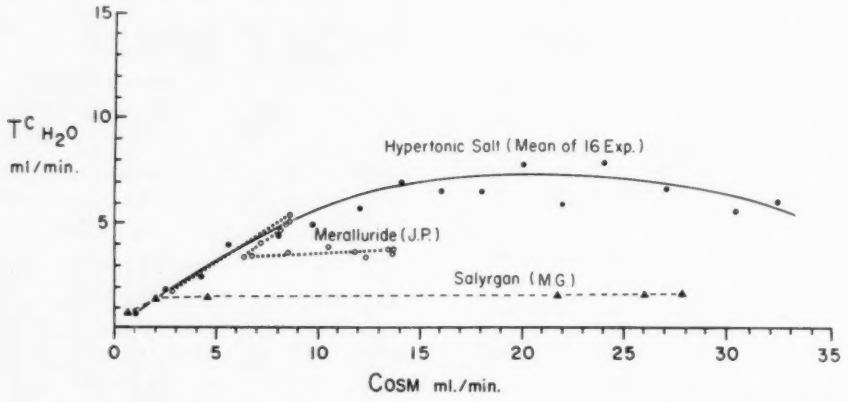

FIG. 2. COMPARISON OF THE 2 PHASES OF A MERALLURIDE DIURESIS WITH THE DIURESIS PRODUCED BY A NONTHEOPHYLLINE-CONTAINING MERCURIAL (SALYRGAN) AND BY A NONSPECIFIC SOLUTE DIURETIC (HYPERTONIC SALT).

The effective renal plasma flow, when measured, varied as the GFR, i.e., there was a prompt fall averaging 31 per cent when the GFR fell after administration of Salyrgan or mercaptomerin and there was a coincident rise averaging 29 per cent when GFR transiently increased with meralluride.

During the mercurial diuresis the increase in sodium and chloride excretion was commensurate with the increase in C_{Osm}. In the majority of experiments potassium excretion fell.

Nonspecific solute diuretics superimposed on organomercurials (*Tables III and IV, Figure 3*). In the 12 experiments in which a nonspecific agent (hypertonic mannitol, hypertonic salt or aminophylline) was superimposed upon a mercurial diuresis, an increase in T^cH_2O was noted in each instance. In 10 of these 12 experiments T^cH_2O increased at least 1.4 ml per minute, with a mean of 2.0 (range, 1.4 to 3.0). This increase in T^cH_2O was associated with a mean increase in C_{Osm} of 15.5 ml per minute (range, 6.4 to 30.5). The in-

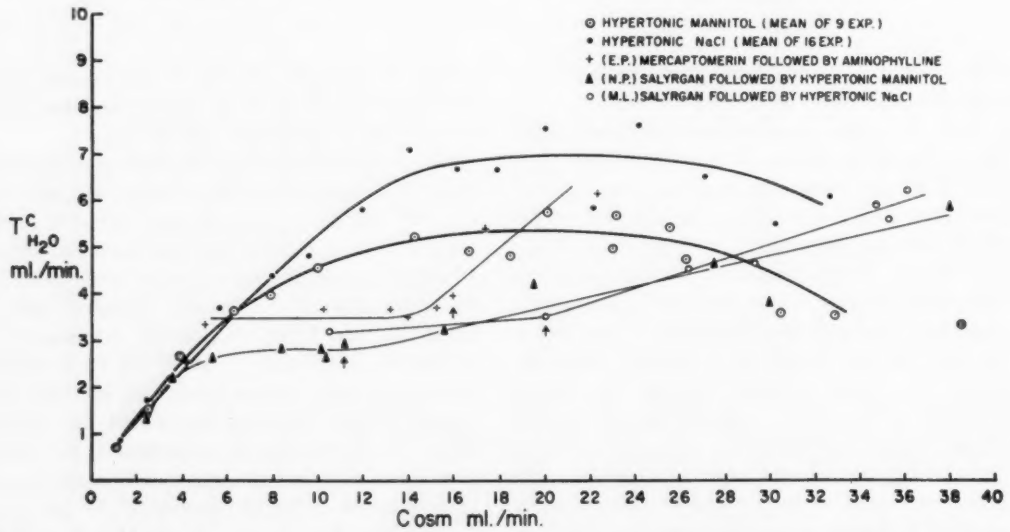

FIG. 3. EFFECT OF A NONSPECIFIC SOLUTE DIURETIC SUPERIMPOSED ON ORGANOMERCURIAL DIURESIS. The arrow indicates the point of administration of the nonspecific agent.

TABLE IV

Effect of nonspecific solute diuretics superimposed on organomercurials on solute excretion and free water reabsorption in hydropenic man

Subject	Mercurial		Solute excretion* (UV/P)	Free water reabsorption (T^cH_2O)
			ml/min	*ml/min*
M.G.	Salyrgan	I	26.0	1.7
		IIa	41.7	3.4
N.P.	Salyrgan	I	11.1	2.9
		IIb	38.1	5.9
A.C.	Salyrgan	I	12.4	3.3
		IIc	21.5	5.1
L.G.	Salyrgan	I	17.4	3.3
		IIc	26.1	4.2
M.L.	Salyrgan	I	20.0	3.5
		IIa	36.3	6.3
B.D.	Mercaptomerin	I	19.7	0.5
		IIb	36.6	2.4
M.P.	Mercaptomerin	I	12.7	0.7
		IIa	26.5	1.2
M.R.	Mercaptomerin	I	10.7	2.1
		IIc	17.8	3.5
E.P.	Mercaptomerin	I	16.0	4.0
		IIc	22.4	6.2
A.S.	Meralluride	I	9.9	3.4
		IIa	23.6	4.8
H.L.	Meralluride	I	8.1	1.7
		IIb	38.6	3.3
R.M.	Meralluride	I	10.0	2.6
		IIb	32.1	4.3

* I represents the period during the maximal mercurial diuresis just prior to the administration of the superimposed agent; II represents the period of peak solute excretion and free water reabsorption with the superimposed agent; a = 2.5% sodium chloride; b = 10% mannitol; c = aminophylline.

crease in T^cH_2O produced by the superimposed solute diuretics was comparable for the three organomercurials.

Reduction in glomerular filtration rate during organomercurial and hypertonic salt diureses (Figure 4). In the experiments in which the blood pressure was suddenly reduced during a mercurial diuresis, GFR decreased a mean of 68 per cent and ERPF a mean of 60 per cent. Coincidentally, a prompt decrease in C_{Osm} occurred, averaging 11.5 ml per minute, but urine osmolality increased only slightly (mean increase, 27 mOsm per kg). As solute excretion diminished, urine osmolality and T^cH_2O values were significantly below those noted at comparable levels of solute clearance as the diuresis was developing.

When the blood pressure was abruptly lowered during the diureses induced by hypertonic salt infusions, GFR and ERPF were reduced an average of 42 and 48 per cent, respectively. Coincidentally, C_{Osm} decreased a mean of 8.7 ml per minute, but with this reduction in solute excretion an appreciable rise in urine osmolality occurred, averaging 237 mOsm per kg. As solute excretion fell, urine osmolality and calculated T^cH_2O values were similar to those observed at comparable C_{Osm}'s during the development of the diuresis. The reductions in solute excretion persisted for approximately 30 minutes in both the mercurial and salt-infusion studies.

DISCUSSION

In those experiments with Salyrgan and mercaptomerin in which an early reduction in solute excretion and T^cH_2O values was observed, a simultaneous fall in filtration rate was noted. Although the recorded mean reduction in GFR of 26 per cent is exaggerated by the abrupt fall in urine flow (18), a modest reduction in filtration rate appears to be responsible for these changes in C_{Osm} and T^cH_2O. The early but transient increase in filtration rate noted with meralluride was probably also exaggerated by the abrupt increase in urine flow from control hydropenic levels. However, this rise in filtration rate may in part explain the transient first phase of a meralluride diuresis in which C_{Osm} and T^cH_2O increased (Figure 2).

Apart from these early minor variations, it is evident from the data that T^cH_2O remains relatively constant during a mercurial diuresis at that level which obtained immediately prior to the development of the major saluresis (Tables I and II, Figure 1). This finding is also apparent in the data presented by Au and Raisz (11) and explains the diverse conclusions drawn by these and other authors concerning the effect of mercurials on the concentrating operation. When the mercurial is administered under conditions of relatively low solute clearance, the persistence of low T^cH_2O levels is reflected as an apparent defect in the concentrating capacity of the kidney (11). However, when the mercurial is administered to a

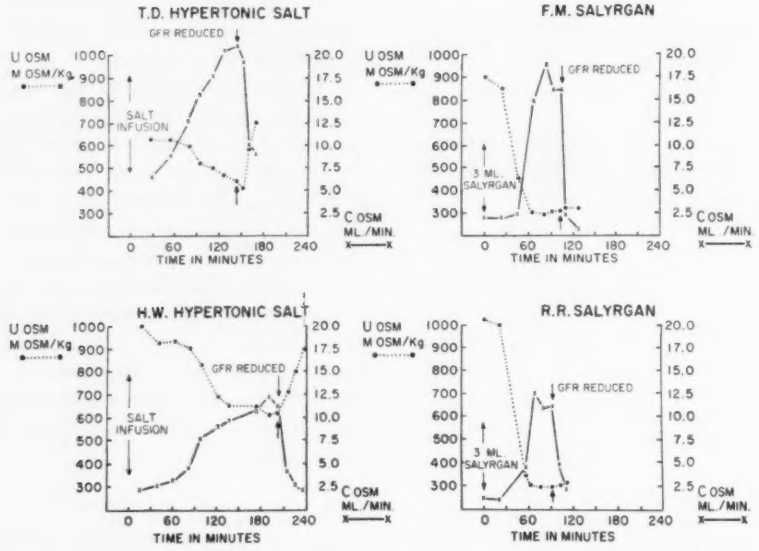

FIG. 4. CHANGES IN URINE OSMOLALITY (U_{Osm}) AND SOLUTE EXCRETION (C_{Osm}) DURING THE DIURESIS PRODUCED BY ORGANOMERCURIALS OR HYPERTONIC SALT INFUSIONS AND AFTER THE EXPERIMENTAL REDUCTION IN GFR. The arrows indicate the time at which GFR and C_{Osm} were reduced.

subject excreting large quantities of solute and extracting appreciable quantities of solute-free water, impaired concentrating capacity is not demonstrable (12, 13).

At very low rates of solute flow, a limiting factor in obtaining a maximal value for T^cH_2O appears to be the meager quantity of water (and solute) reaching the collecting duct. The early increase in T^cH_2O noted with very small increments in solute clearance in both the control studies and several of the mercurial experiments (Tables I and III, N.P., H.B., A.S., R.M.; Figure 1, N.P.) may therefore be attributed to the increased quantity of water presented to this site. However, on the basis of mercurial data, it is apparent that the presentation of increasing quantities of water (and solute) to the collecting duct does not in itself assure the development of a maximal T^cH_2O. No substantial evidence is available to suggest that mercurials inhibit the action of antidiuretic hormone or limit tubular permeability to water (1, 5, 11, 19–21). Therefore, the rising T^cH_2O in the control studies, contrasted with the constancy of this parameter during a comparable mercurial diuresis, suggests that during the former more osmotically active particles were present within the medullary interstitium. An agent which inhibits proximal tubular salt and water reabsorption would present more salt to the loop of Henle, a fraction of which could be transferred into the interstitial fluid (14). By this means, additional osmotic activity would be provided for the extraction of water from the isosmotic fluid in the adjacent collecting duct. At comparable levels of solute excretion, salt as the loading solute would be expected to present more absorbable particles than mannitol, and thereby produce a higher maximal value for T^cH_2O. Such was the case in the control studies reported here and apparent in the data reported by others in the hydropenic hamster and rat (14). Accordingly, peak T^cH_2O values will be achieved in hydropenic man when the dynamics of flow within the loop provide a maximal quantity of solute for transport into the interstitium and, simultaneously, adequate quantities of isosmotic fluid reach the collecting duct.

The failure of additional solute to gain access to the medullary interstitium during the course of a mercurial diuresis tends to exclude the likelihood that mercurial agents exert their sole inhibitory effect within the proximal tubule. The inhibition of salt absorption at this site would be expected to

present at least as much solute to the ascending limb of the loop of Henle as mannitol and salt infusions at comparable levels of solute excretion. Progressive inhibition of salt reabsorption in the ascending limb of the loop of Henle during a mercurial diuresis might explain the failure of T^cH_2O to rise. However, the constancy of T^cH_2O in the experiments in which this parameter was elevated prior to the mercurial diuresis, noted here and by others (11) argues against this hypothesis. Furthermore, this explanation is not consistent with the increase in T^cH_2O produced by the administration of a nonspecific solute load during a mercurial diuresis (Tables III and IV, Figure 3). This finding suggests that when additional quantities of salt are presented to the loop of Henle during a mercurial diuresis, additional solute can be transported into the interstitial fluid. Moreover, progressive inhibition of salt reabsorption at this site conflicts with the observations in hydrated man that free water clearance remains relatively fixed as a mercurial diuresis develops (10). Although these data do not exclude some minor inhibition of salt reabsorption at the proximal segment or at the ascending limb of Henle's loop, they do weaken the possibility that the major site of action of organomercurials resides at either of these loci.

Another explanation for the failure of salt to be deposited in the interstitial fluid during a mercurial diuresis would presume that organomercurials enhance medullary blood flow and so limit the trapping characteristics of the medullary circulation. This hypothesis is not consistent with the observation that nontheophylline-containing mercurials tend to decrease total renal plasma flow. It seems unlikely that opposite qualitative changes in renal plasma flow and medullary flow would occur simultaneously. Pyrogenic reactions, which enhance renal plasma flow and presumably medullary blood flow, consistently reduce T^cH_2O (22, 23), an action different from that produced by mercurials.

A hypothesis compatible with the observations reported here would place the major inhibitory action of mercurials at a site distal to the loop of Henle. Such an action would explain the relative constancy of T^cH_2O during a mercurial diuresis, since the major increase in the flow of isosmotic fluid through the collecting duct would derive from a site beyond the loop of Henle. The effective osmotic gradient between interstitial and collecting duct fluid during any steady state is dependent upon the balance established between solute supply to the interstitium from the ascending limb and water supply from the collecting duct. For each level of solute supply there apparently exists a critical rate of collecting duct flow which will provide a quantity of water for back diffusion adequate to dissipate interstitial fluid tonicity. Without changes in loop solute supply, an increase in collecting duct flow up to this critical rate may produce a rise in T^cH_2O (Tables I and III, N.P., H.B., A.S., R.M.; Figure 1, N.P.) Beyond this critical rate, after medullary tonicity is largely dissipated, it appears that any increase in the flow of isosmotic collecting duct fluid will not in itself measurably augment the T^cH_2O value. This proposal is consistent with the increase in T^cH_2O produced by a superimposed nonspecific solute diuretic during a mercurial diuresis (Tables III and IV, Figure 3) or by the simultaneous administration of a nonspecific agent (13). Either technique would provide additional absorbable solute for loop transport into the interstitium during the mercurial diuresis so that more osmotically active particles would be available to enhance the back diffusion of water from the collecting tubule.

A distal site of mercurial action would also explain why a modest increment in C_{osm} at the onset of the diuresis may be associated with a marked fall in urine osmolality (Table II, M.G.; Figure 4, R.R. and F.M.) In this view, a mercurial diuresis provides additional isotonic fluid to the collecting duct without appreciably increasing the quantity of solute presented to the loop of Henle. The extraction of a small additional quantity of solute-free water at the collecting duct at this time will rapidly attenuate medullary and, therefore, urinary concentration. This postmercurial diminution in medullary tonicity would be most conspicuous when control rates of loop solute flow were low or further reduced by the spontaneous reduction in filtration rate noted after the administration of nontheophylline-containing organomercurials.

This hypothesis can also explain the results obtained when filtration rate was reduced so that solute clearance fell comparably during a mercurial diuresis or a diuresis produced by a salt in-

fusion. Insofar as the diuresis produced by a salt infusion probably derives from a proximal source, it would be expected that loop solute supply would increase as the diuresis develops. When filtration rate is decreased, loop solute supply will return to the same levels which obtained at similarly low flow rates during the development of the diuresis. Therefore, urine osmolality will rise to levels commensurate with reduced levels of solute excretion. On the other hand, during the development of a mercurial diuresis, it has been hypothesized that loop solute supply is not augmented. When the filtered load is reduced during this state, loop solute supply would be expected to fall below those levels which obtained at similarly low flow rates during the development of the diuresis. This fall in loop solute supply might explain the failure of urine osmolality to rise.

The two-phase response produced by meralluride can best be explained by an early and transient increase in the quantity of salt presented to the ascending limb by the theophylline contained within meralluride, followed by a typical organomercurial diuresis. This dual effect would agree with previous observations in hydrated man where the theophylline contained in meralluride also produced an immediate but transient diuresis qualitatively different from that produced by the organomercurial itself (10).

The conclusions drawn from these experiments concerning the site of action of organomercurials in hydropenic man are consistent with previous reports from this laboratory which suggested that the organomercurials exert a major inhibitory influence on an isosmotic salt-absorptive process beyond the ascending limb of the loop of Henle (10).

SUMMARY

1. In the course of a nonspecific solute diuresis in hydropenic man T^cH_2O rises progressively as C_{osm} increases. With hypertonic salt and aminophylline, T^cH_2O reaches a somewhat higher peak than with mannitol.

2. During an organomercurial diuresis T^cH_2O remains relatively fixed at whatever level prevailed prior to the development of the major solute diuresis.

3. The administration of a nonspecific solute diuretic during the organomercurial diuresis increases T^cH_2O.

4. When filtration rate is experimentally reduced in the course of a mercurial diuresis or of a comparable diuresis produced by a salt infusion, urine osmolality rises appreciably only in the salt-infused subjects.

5. These findings suggest a disparity in the supply of solute to the loop of Henle, at every level of solute excretion, between a mercurial diuresis and a comparable diuresis produced by a nonspecific solute diuretic. This disparity supports the hypothesis that the major action of organomercurials resides at a site distal to the loop of Henle.

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URIC ACID METABOLISM IN PSORIASIS

By ARTHUR Z. EISEN* AND J. E. SEEGMILLER

(From the Dermatology Branch, National Cancer Institute, and The National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.)

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Hyperuricemia has been noted to occur in approximately 30 to 40 per cent of patients with psoriasis (1–5). The cause of the hyperuricemia and its possible relation to the psoriatic process has received very little attention.

Uric acid synthesis has been evaluated in man by determining the turnover of injected isotopically labeled uric acid as well as by the incorporation of isotopically labeled glycine into urinary uric acid. Observations made on subjects with primary gout have shown that there is a prompt incorporation of glycine-1-C^{14} or glycine-N^{15} into urinary uric acid (6–13) while in gout secondary to proliferative disorders of the hemopoietic system, isotopic incorporation is delayed presumably because the major pathway of uric acid synthesis involves the incorporation of glycine into tissue purines with their slower rate of turnover (14, 15).

In psoriasis, the skin lesion is a result of hyperplasia of the epidermis in which the rate of cellular reproduction is accelerated to such an extent that maturation of the epidermal cells and the process of normal keratinization do not occur (16). By analogy with the hyperuricemia seen in myeloproliferative diseases, it is possible that the hyperuricemia of psoriasis may reflect an increased nucleic acid turnover resulting from the marked acceleration of epidermal proliferation that occurs in this disease.

The present study was undertaken to determine whether the pattern of glycine-1-C^{14} incorporation into urinary uric acid in psoriasis resembles that seen in primary or in secondary gout. Uric acid-N^{15} was used to obtain information on the dynamics of the body urate pool and to provide an independent parameter of uric acid synthesis.

METHODS

Studies were performed on 5 patients with active psoriasis, on 1 patient with gout and psoriasis and on 2 control subjects. The patient with gout and psoriasis (F.M.) had a history of frequent attacks of acute arthritis which responded to colchicine. A synovial biopsy of his left knee revealed characteristic deposition of urate crystals. The control subjects had no family history or stigmata of either psoriasis or gout. Remission of psoriasis occurred in one patient (L.P.) following a severe drug eruption resulting from the oral administration of chloroquin phosphate. Within 2 weeks after the drug had been discontinued, complete healing of the skin had occurred and no lesions of psoriasis were present. The repeat glycine-1-C^{14} incorporation study was carried out approximately 10 weeks after the last dose of chloroquin had been administered, while the psoriasis was still in complete remission.

All subjects were hospitalized throughout the studies and were maintained on a standard 2,600 calorie diet, essentially free of purines and containing 70 g of protein, 350 g of carbohydrate and 100 g of fat. At least 5 days were allowed for equilibration on the diet before the studies were performed. Patients received no medication during the study. The clinical diagnosis of psoriasis in each case was confirmed by skin biopsy. Renal function was assessed by routine urinalysis, blood urea nitrogen and phenolsulfonphthalein excretion.

Daily 24-hour urine collections were preserved with toluene and stored at room temperature. Serum and urinary uric acid determinations were performed by means of the enzymatic spectrophotometric assay of Praetorius and Poulsen (17) as modified by Liddle, Seegmiller and Laster (18). The upper range of normal for the 24-hour urinary uric acid excretion on the standard purine-free diet was considered as 600 mg per day (13). A serum urate value of 6.0 mg per 100 ml for males and 5.0 mg per 100 ml for females by the colorimetric method of Archibald (19), corresponding to a value of 6.2 mg per 100 ml for males and 5.2 mg per 100 ml for females by the enzymatic method, was taken as the upper limit of normal. These values are only slightly more than 1 standard deviation from the mean for the serum urate values of a large group of normal subjects combined from the literature, 5.1 ± 0.98 mg per 100 ml for 969 males and 4.0 ± 0.93 mg per 100 ml for 168 females (20). Extension of the range of normal values to include 2 standard deviations above the mean (7.0 mg per 100 ml for males and 5.9 mg per 100 ml for females) would result in a substantial number of patients with conditions associated with "hyperuricemia" being included in the upper range of normal.

Glycine-1-C^{14} with a specific activity of 1.0 mc per mmole (Volk Radiochemical Corporation of Chicago)

* Present address: Dept. of Medicine, Rochester School of Medicine and Dentistry, Rochester, N. Y.

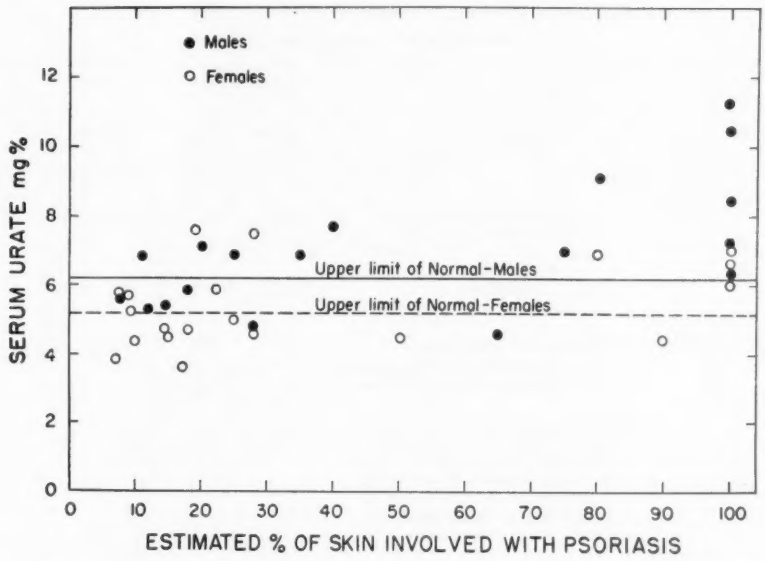

FIG. 1. SERUM URATE VALUES CORRELATED WITH THE ESTIMATED PERCENTAGE OF SKIN INVOLVED WITH PSORIASIS. The correlation coefficient R = 0.567. The above determinations of uric acid were performed using the enzymatic spectrophotometric method. The values shown for the upper limits of normal of 6.2 mg % for males and 5.2 mg % for females correspond to values obtained by the Archibald colorimetric method of 6.0 and 5.0 mg %, respectively (19).

was given orally in a single dose of 5 or 10 μc with the breakfast milk. Simultaneously, 36 mg of uric acid 1,3-N^{15},[1] containing 28.6 atom per cent excess of N^{15}, was administered intravenously as the lithium salt to measure the urate pool and to allow corrections to be made for altered dynamics of this pool. Samples of uric acid for intravenous injection were sterilized by filtration of the lithium salt in 5 per cent glucose solution through a 0.45 μ Millipore filter.

Uric acid was isolated from the urine either by adsorption onto activated charcoal (21) or by precipitation as a copper salt when urine volumes exceeded 1,500 ml per day (22), and purified by repeated crystallization from lithium carbonate solution by addition of acetic acid (23). The radioactivity was determined using an end-window gas-flow counter (Nuclear Chicago), from planchets prepared at infinite thickness. All specific activity values were corrected to a standard dose of 5 μc of glycine-1-C^{14}. The N^{15} content of the uric acid was determined in a 60° Consolidated Nier mass spectrometer following preliminary Kjeldahl digestion (24).

RESULTS

Hyperuricemia was associated with psoriasis in 50 per cent of 38 patients with this disease seen at the Clinical Center during the past 18 months. In 10 of the 18 males the serum urate values ranged from 6.6 to 11.3 mg per 100 ml. The values for seven of these patients are undoubtedly lower than their usual levels by approximately 1.0 mg per 100 ml, since they were being maintained on a purine-free diet at the time the determination was made (13). The three remaining males of this group showed serum urate values above 7.1 mg per 100 ml. Nine of 20 female patients had serum urate levels ranging from 5.4 to 7.6 mg per 100 ml. None of these 38 patients had a family history of gout and there was no clinical or laboratory evidence of associated renal, hepatic or hematologic disease which might result in hyperuricemia. Fourteen patients had a nongouty type of arthritis which is frequently associated with the psoriatic process and of these, eight had elevation of the serum urate. In this small series, patients with extensive involvement of the skin tended to have a higher incidence of hyperuricemia (Figure 1), suggesting that the elevation of the serum urate may be associated with excessive epidermal proliferation.

[1] We are indebted to Miss Jean D. Benedict for providing the uric acid-N^{15}.

TABLE I

Data on subjects given glycine-1-C^{14}

Subject, Age, Race, Sex	Wt	Clinical summary	Serum uric acid: Before diet	Serum uric acid: After 7 days on diet	Urinary uric acid	Renal function*: BUN	Renal function*: PSP (2 hr)
	kg		*mg%*	*mg%*	*mg/day ± SD†*	*mg%*	*%*
M.G. 38, W, ♀	78.5	Control subject, small basal cell carcinomata on face	3.4	3.2	357 ± 51	11	67
P.D. 19, W, ♂	65.5	Normal volunteer	4.5	4.5	459 ± 56	14	72
B.P. 30, W, ♂	77.5	Psoriasis of 10 yrs duration, approximately 80% skin involvement, no joint disease	9.1	8.5	601 ± 36	11	78
J.F. 20, W, ♂	66.7	Psoriasis of 7 yrs duration, psoriatic arthritis for 2 yrs, no acute attacks of arthritis, no response of arthritis to colchicine or Probenicid, 100% skin involvement	9.7	6.8	773 ± 78	8	76
W.M. 54, W, ♂	71	Psoriasis of 25 yrs duration, approximately 75% skin involvement, no joint disease	7.1	6.9	631 ± 50	12	82
W.J. 31, W, ♂	95.5	Psoriasis of 13 yrs duration, approximately 35% skin involvement, no joint disease	5.7	5.8	557 ± 40	11	84
L.P. 73, W, ♀	70.2	Study 1: psoriasis active and of 25 yrs duration, approximately 50% skin involvement, no joint disease	4.9	4.2	312 ± 32	8	73
		Study 2: psoriasis in complete remission	4.6	4.8	223 ± 17	10	
F.M. 56, W, ♂	84.4	Psoriasis of 13 yrs duration, 9 yr history of acute gouty arthritis, approximately 60% skin involvement, chronic alcoholic, 20% BSP retention	8.7	5.6	548 ± 44	11	74

* BUN = blood urea nitrogen; PSP = phenolsulfonphthalein; BSP = sulfobromophthalein.

† SD refers to the standard deviation of the mean values for urinary uric acid excretion during the period of study following the administration of glycine-1-C^{14}.

The pertinent clinical data on the subjects included in this study are presented in Table I. Included is an estimate of the degree of skin involvement, as determined by the method of Evans and associates (25), at the time of isotope administration. Three of the patients had greater than 70 per cent and two had less than 50 per cent of their skin involved with psoriasis. In most subjects the serum urate values after 7 days on the purine-free diet were only slightly lower than the serum levels prior to initiation of the diet. In Subjects J.F. and F.M., however, the serum uric acid values decreased quite markedly after initial institution of the diet but thereafter remained at a constant level.

The specific activities of C^{14} in urinary uric acid after administration of a tracer dose of glycine-1-C^{14} are shown in Figure 2. The specific activity of uric acid obtained from control subjects

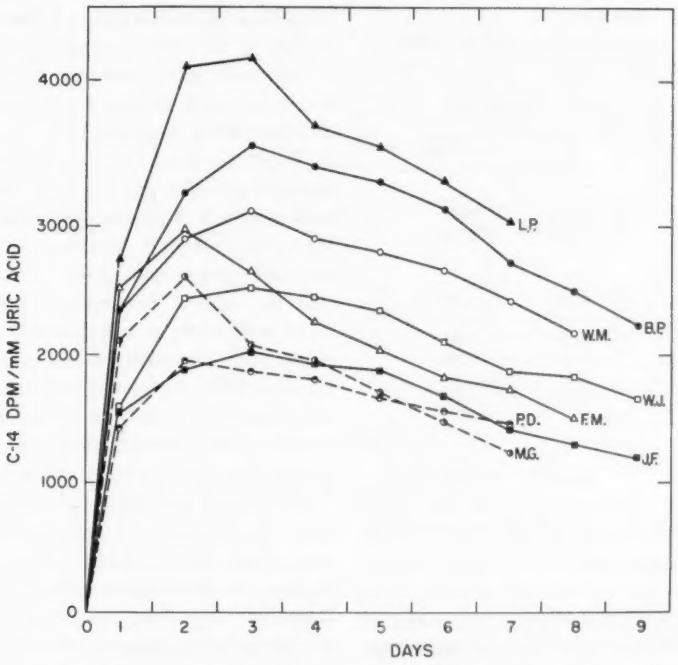

FIG. 2. SPECIFIC ACTIVITY VALUES (DISINTEGRATIONS PER MINUTE PER MILLIMOLE OF URINARY URIC ACID) IN CONTROL AND PSORIATIC SUBJECTS FOLLOWING ORAL ADMINISTRATION OF GLYCINE-1-C^{14}. B.P., W.M. and J.F. were patients with psoriasis who excreted excessive quantities of uric acid in their urine (i.e., greater than 600 mg per day). L.P. and W.J. represent patients with psoriasis who had a normal urinary excretion of uric acid. F.M. represents a patient with psoriasis and gout. The two control subjects studied were P.D. and M.G. All values are normalized to a standard dose of 5 μc of glycine-1-C^{14}.

P.D. and M.G. was within the range that has been demonstrated by previous investigations for normal control subjects (13). In the five nongouty psoriatic patients, the specific activities rose quite rapidly, reaching a maximum on the third day, followed by a gradual decline in isotope concentration. In Patients B.P. and W.M., who excreted excessive amounts of uric acid, the maximal specific activities were considerably higher than those of the control subjects. Patient J.F., however, who also produced excessive amounts of uric acid in his urine, showed a maximum specific activity within the range of the control subjects. The fact that he showed no extrarenal disposal of uric acid (Table II) may account in part for his high urinary production of uric acid.

Patient L.P. is of particular interest; the specific activity of C^{14} in her urinary uric acid was the highest of all the subjects studied, reaching a maximum of 4,291 dpm per mmole of uric acid on the third day after the isotope was administered. At the time this patient was studied, both her serum and urinary uric acid levels were well within normal limits. This is most unusual, since excessively high concentrations of isotope in the uric acid are most often associated with elevated serum and urinary levels (12, 13). In W.J., who also had normal serum and urinary uric acid levels, the pattern of incorporation closely resembles that of the other psoriatic patients.

The association of psoriasis with gout has been reported (5, 26). Patient F.M. demonstrated such an association, and was studied in an effort to determine whether his pattern of glycine incorporation into urinary uric acid resembled that of primary gout or psoriasis. The peak specific ac-

TABLE II

Seven-day recovery of isotope in urinary uric acid

	Per cent of administered uric acid-N^{15} (a)	Per cent of administered glycine-1-C^{14}	
		Uncorrected (b)	Corrected (b/a×100)
Controls			
M.G.	82.1	0.27	0.34
P.D.	77.0	0.29	0.37
Psoriasis			
B.P.	64.4	0.70	1.09
J.F.	99.3	0.51	0.52
W.M.	81.5	0.69	0.84
W.J.	74.1	0.45	0.61
L.P.*		0.42	
L.P.†	53.0	0.17	0.32
Psoriasis and gout			
F.M.	70.2	0.48	0.69

* Study 1: psoriasis active; patient did not receive uric acid-N^{15}.

† Study 2: psoriasis in remission.

tivity of C^{14} in this subject's urinary uric acid was reached on the second day and declined more rapidly than in the other psoriatic patients. This pattern tends to resemble more closely that found in subjects with primary gout rather than the pattern shown by the other psoriatic patients studied.

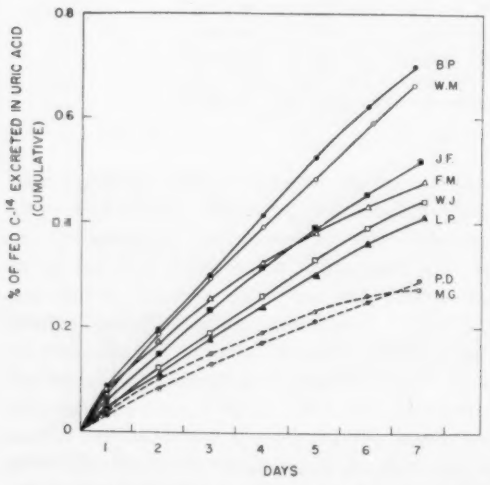

FIG. 3. CUMULATIVE INCORPORATION OF GLYCINE-1-C^{14} INTO URINARY URIC ACID OF SUBJECTS PRESENTED IN FIGURE 1. B.P., W.M. and J.F. are patients with psoriasis who excreted excessive amounts of urinary uric acid. L.P. and W.J. are patients with psoriasis who had a normal urinary excretion of uric acid. F.M. is a patient with psoriasis and gout. P.D. and M.G. are control subjects.

The cumulative recovery of isotope in the urinary uric acid during the 7 days following the administration of glycine-1-C^{14} is shown in Figure 3. In all five nongouty, psoriatic patients excessive incorporation of glycine-1-C^{14} into urinary uric acid is clearly demonstrated when compared with the control subjects; this is also true for F.M., the psoriatic patient with gout. It was not surprising that the highest percentage incorporation values were seen in those subjects who excreted abnormally large amounts of uric acid in their urine. Unexpectedly, however, Patients L.P. and W.J., who excreted normal quantities of uric acid, nevertheless incorporated excessive quantities of the isotope into their urinary uric acid.

Substantial uricolysis has been demonstrated to occur in man (*27*). Furthermore, with hyperuricemia there is a possibility of urate deposition in tissues. Such extrarenal disposal of uric acid has been shown in some gouty patients to alter substantially the cumulative recovery values obtained by measurements of urinary uric acid alone (13). The magnitude of such extrarenal disposal of uric acid is indicated by the percentage of the injected uric acid-N^{15} which is recovered in the urine. Table II demonstrates the wide variation in this parameter among these patients.

By correcting the cumulative C^{14} recovery values for the fraction of the daily uric acid production that fails to appear in the urine, it is possible to estimate the total amount of C^{14} incorporated into uric acid within the body. These values, shown in Table II, serve to emphasize the degree of overincorporation of isotope demonstrated by the psoriatic subjects when compared with the control subjects.

In addition to the evaluation of extrarenal disposal of uric acid, the use of uric acid 1,3-N^{15} permitted an estimation of the urate pool size and turnover which is shown in Table III. The urate pool size in these subjects seemed to be a reflection of their serum urate levels. Calculations indicated that there was no solid phase urate in their miscible pools (28). Although the turnover values for the patients with psoriasis were greater than those of both control subjects, other investigators (6, 29) have found turnover values in some control subjects that overlap with the values we obtained

TABLE III

Miscible urate pool size and turnover

Subject	Mean serum uric acid	Urate pool size (A)	Turnover rate (K)	Turnover (KA)	Excretion of uric acid (E)	Per cent of turnover excreted (E/KA ×100)
	mg%	*mg*	*pools/day*	*mg/day*		
Control						
M.G.	3.2	687	0.738	506	357	71.0
P.D.	4.5	1,162	0.559	650	459	70.6
Psoriasis						
B.P.	8.5	1,860	0.435	809	601	74.0
J.F.	6.8	1,221	0.621	772	773	100
W.M.	6.6	1,348	0.551	743	637	87.1
W.J.	5.8	1,381	0.490	677	549	82.5
L.P.*	4.2				312	
L.P.†	4.7	880	0.454	400	223	55.7
Psoriasis and gout						
F.M.	5.6	1,297	0.585	749	556	73.2

* Study 1: psoriasis active; patient did not receive uric acid-N^{15}.
† Study 2: psoriasis in remission.

for the patients with psoriasis. The last column of Table III provides an independent evaluation of extrarenal disposal which is in general agreement with recovery values shown in Table II.

Effect of remission of psoriasis on uric acid biosynthesis. The occurrence of a remission of psoriasis in Patient L.P. provided an opportunity to evaluate the extent to which the psoriatic process may have contributed to the abnormal glycine incorporation. A repeat study was performed 2 months after complete clearing of the skin (4 months after completion of the initial study). As shown in Figure 4, the specific activity of C^{14} in urinary uric acid had decreased markedly with remission of the psoriasis, and the pattern of incorporation closely resembles that of the control subjects. The cumulative percentage incorporation values were also within the same range as those shown by the control subjects.

DISCUSSION

In previous studies of the pattern of incorporation of either glycine-1-C^{14} or glycine-N^{15} into urinary uric acid, most patients with primary gout, as well as most normal subjects (13), have shown a maximal specific activity on the first or second day. An occasional normal subject, however, has shown a maximal specific activity on the third or even the fourth day. By contrast, patients with gout secondary to myeloproliferative disorders studied with glycine-N^{15} showed low specific activities during the first few days with a progressive increase to maximal values on the tenth to fourteenth day (14, 15). One patient with agnogenic myeloid metaplasia studied with glycine-1-C^{14} (30) showed a maximal specific activity in urinary uric acid on the third day; however, the isotope con-

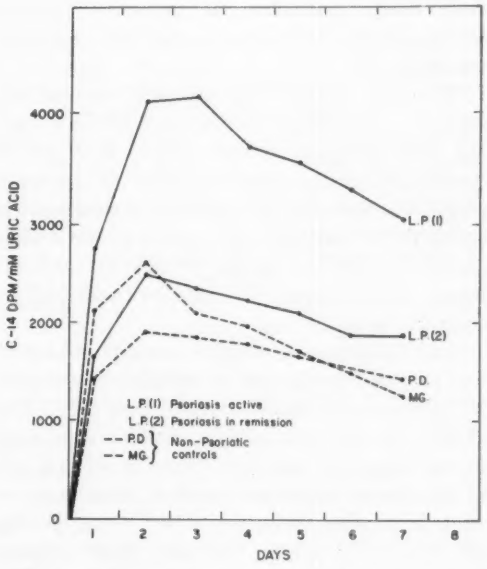

FIG. 4. SPECIFIC ACTIVITY VALUES (DISINTEGRATIONS PER MINUTE PER MILLIMOLE OF URINARY URIC ACID) IN CONTROL SUBJECTS AND IN PATIENT L.P.

centration during the ensuing days declined less rapidly than in patients with primary gout or in the control subjects, with evidence of a secondary phase of C^{14} incorporation into uric acid which was maximal on the eleventh to thirteenth day. The fact that the life span of the circulating granulocytes is approximately 13 days suggests that the turnover of granulocyte nucleic acid may be related to the delayed appearance of isotope in urinary uric acid (31).

The pattern of glycine-1-C^{14} incorporation into uric acid in the psoriatic patients was consistently intermediate between that shown by patients with primary gout and that shown by patients with secondary gout. The fact that the turnover time of the stratum corneum of the epidermis in individuals with psoriasis is 3 to 4 days as compared with approximately 28 days in nonpsoriatic individuals (32), suggests that the peak enrichment of uric acid seen on the third day may, in part, be related to a more rapid turnover of nucleic acids involved in this proliferative process. In addition, the possibility exists that the relative amounts of uric acid formed directly from nucleotides has been enhanced by the psoriatic process. Patient F.M., with both gout and psoriasis, showed a maximal specific activity on the second day and therefore more nearly resembled the pattern of labeling seen in patients with primary gout than in patients with psoriasis.

All of the patients with psoriasis showed substantially greater total recoveries of C^{14} in the urinary uric acid over a 7-day period than did the control subjects. Correction of the glycine incorporation values for the fraction of administered uric acid-N^{15} that failed to appear in the urinary uric acid (Table II) serves to emphasize this difference between patients with psoriasis and the control subjects.

An independent measure of uric acid production was provided by the rate of turnover of uric acid-N^{15} administered intravenously (Table III). Previously reported studies have shown a wide range in the values for the daily turnover of uric acid in the normal individual, with a mean turnover value in 13 such subjects of 685 ± 88 mg per day (6, 13, 21, 27, 29). The daily urate turnover in the psoriatic patients was for the most part within the upper range of normal values except for Patient B.P. in whom this parameter was significantly elevated. The turnover of uric acid as measured by uric acid-N^{15} did not show a close correlation with the amount of glycine-1-C^{14} incorporated into urinary uric acid. In general, the incorporation of C^{14} into uric acid was greater than one would expect from the urate turnover values alone, suggesting that the increased incorporation of isotopically labeled glycine into urinary uric acid occurred in the face of normal or only moderate elevations in the size of the urate miscible pool and in its daily turnover.

The presence of high specific activity values as well as elevated cumulative isotopic incorporation values in the two psoriatic patients (L.P. and W.J.) who excreted normal amounts of uric acid in their daily urine, is of particular interest. It would appear that in some patients with psoriasis, such as L.P. and W.J., metabolic processes are so altered as to enhance isotopic incorporation into uric acid without a proportionate increase in net synthesis of uric acid. A similar situation has been observed in some patients with primary gout and a mechanism proposed whereby dietary glycine enters urinary urate through prompt degradation of a larger fraction of newly formed purine nucleotides without the delay that results from incorporation into nucleic acids (9). This could account for a disproportionately high glycine incorporation in the face of a moderate increase in total uric acid synthesis. Alternative mechanisms that could account for an increased incorporation of glycine into uric acid without a proportionate increase in uric acid synthesis include: *a*) a reduction in the size of the glycine pool which would result in less dilution of the administered isotopically labeled glycine and hence a higher specific activity of that glycine which does become incorporated into uric acid, or *b*) a reduction in the turnover rate of the glycine pool so that the rate of disposal of glycine by pathways other than purine synthesis is diminished, which thereby results in a larger fraction of the glycine-1-C^{14} remaining available for purine synthesis. Just which of the many possible explanations is operative in psoriasis has not been determined.

A diminished uricolysis has been postulated as a cause of hyperuricemia (33). The essentially complete recovery in the urine of intravenously administered uric acid-N^{15} in Patient J.F. indicates the absence of appreciable uricolysis in this

patient. Further support for this conclusion is provided by the remarkable agreement of the uric acid turnover with the urinary production of uric acid in this patient. Similar results have been reported in one gouty subject (34) and have been noted as an inconstant finding in two other subjects (35). Evidence has been presented that uricolysis is the result of bacterial action on the uric acid present in intestinal secretions (*27*). A reasonable explanation for the apparent absence of uricolysis in Patient J.F. would be the absence of uricolytic bacteria in the intestinal flora at the time he was studied, even though no antibacterial therapy was given.

The data shown in Figure 4 in which the glycine incorporation values decreased to essentially the normal range, when the study was repeated after a remission of the active psoriatic process, suggests that this abnormality in glycine-1-C^{14} incorporation into urinary uric acid was related in some manner to the active psoriatic process rather than to a persistent defect in the utilization of glycine for uric acid synthesis.

It is of particular interest that although these patients showed an increased glycine incorporation into urinary uric acid and, in some, excessive uric acid production was present, these individuals, with the exception of Patient F.M., showed no evidence of gouty arthritis.

SUMMARY

1. Nineteen of 38 patients with psoriasis were found to have an associated elevation of the serum uric acid. Patients with extensive involvement of the skin tended to have a higher incidence of hyperuricemia.

2. Five patients with psoriasis all showed a greater than normal incorporation of glycine into urinary uric acid.

3. The pattern of incorporation of C^{14} into urinary uric acid tended to differ from that seen in control subjects and appeared to be intermediate between that shown by patients with primary gout and those with secondary gout. The specific activity of C^{14} in urinary uric acid reached a maximum on the third day after administration of the isotope in all the patients with psoriasis, while one patient with gout and psoriasis showed a maximal specific activity on the second day.

4. The abnormal labeling of urinary uric acid returned to normal after clinical remission of psoriasis in one patient.

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AN ANALYSIS OF FACTORS AFFECTING THE MEASUREMENT OF PULMONARY DIFFUSING CAPACITY BY THE SINGLE BREATH METHOD *

By JOHN B. CADIGAN,† ASHER MARKS,‡ MARJORIE F. ELLICOTT, ROBERT H. JONES † AND EDWARD A. GAENSLER §

(From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, and the Thoracic Surgery Service, Boston City Hospital; the Departments of Medicine and Surgery, Harvard Medical School; and the Thoracic Services, Departments of Medicine and Surgery, Boston University School of Medicine, Boston, Mass.)

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The diffusing capacity of the lungs (D_L) is defined as the rate of a gas transferred, divided by the difference in the mean partial pressure of that gas across the alveolar-capillary "membrane." Direct determination of the diffusing capacity for oxygen ($D_{L_{O_2}}$) has been limited by technical difficulties and by the problematical calculation of the mean oxygen pressure on the capillary side of the pulmonary membrane. Measurement of D_L for carbon monoxide ($D_{L_{CO}}$) has been preferred because the affinity of CO for hemoglobin is so great that plasma tension or "back pressure" has been considered negligible and therefore disregarded (1).

The technique which Krogh (2) introduced for determination of $D_{L_{CO}}$ involved measurement of alveolar CO pressure ($\bar{P}_{A_{CO}}$) at the beginning and end of breath-holding. Gas containing CO was inhaled and a small portion exhaled immediately for determination of the initial CO pressure ($\bar{P}_{A_{CO_i}}$). A second sample was delivered at the end of the breath-holding period for measurement of the final CO pressure ($\bar{P}_{A_{CO_f}}$). Also determined were the volume of the lungs at which diffusion occurred (V_A) and the barometric pressure (B). If $\bar{P}_{A_{CO}}$ decays exponentially with time (t) during breath-holding, and if the plasma back pressure is zero, then:

$$D_L{}^1 = \frac{V_A}{B - 47} \times \left[\ln \frac{(\bar{P}_{A_{CO_i}})}{(\bar{P}_{A_{CO_f}})} \right] \times \frac{1}{t} \quad [1]$$

The suggestion by Forster, Fowler, Bates and Van Lingen (3) to introduce nondiffusible helium as a tracer in the inspired gas has eliminated the need for initial "alveolar sampling." $\bar{P}_{A_{CO_i}}$ of the previous equation can then be calculated from the helium concentration (F_{He}) of the inspired and expired gases:

$$\bar{P}_{A_{CO_i}} = P_{I_{CO}} \times \frac{F_{E_{He}}}{F_{I_{He}}} \quad [2]$$

This method is attractive for clinical estimation of the diffusing capacity because it is bloodless, and the entire test with analyses requires only a few minutes once the residual volume has been determined. However, during studies in our laboratories beginning in 1954 some disturbing facts emerged concerning this method. We noted that: *a*) values for the single breath D_L in any given case were always larger than those obtained by either the $D_{L_{CO}}$ or $D_{L_{O_2}}$ steady state methods (4); *b*) certain patients with reduced steady state $D_{L_{O_2}}$ and $D_{L_{CO}}$ had normal values by the single breath test (4); *c*) the $D_{L_{CO}}$ declined slightly with repeated determinations suggesting that mean pulmonary capillary CO pressure may not be negligible; and *d*) duplicate or multiple determinations often did not check satisfactorily. The largest deviations occurred when breaths were held at different lung volumes, and increases in lung volume of 50 per cent often caused a 50 per cent increase

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[1] Normally a subscript of CO or O_2 is used to indicate the gas to which the measurement applies. In this article, which is mainly concerned with CO, the subscript is omitted and can be assumed to be CO unless otherwise stated.

of the single breath D_L (4, 5). Such differences were first observed by Krogh (2) but recently Forster and associates (3, 6) and Ogilvie, Forster, Blakemore and Morton (7) have not been able to find significant differences in D_L due to changes in lung volume.

The purpose of the present study was, therefore: *a*) to study the relationship between alveolar volume (V_A) and D_L; b) to study the effects of plasma CO back pressure on the values obtained by the single breath technique; and *c*) to consider the reasons for the numerical differences between D_L values obtained by the single breath and steady state techniques.

METHODS

The single breath D_L was determined according to Krogh with the modifications of Forster and co-workers (3). Seated subjects inhaled a mixture containing approximately 0.3 per cent CO, 20 per cent O_2, 10 per cent He, and 70 per cent N_2 through a large-bore, four-way valve from a box-balloon spirometer designed for this purpose.[2] The inspired volume was recorded by the spirometer attached to the box. Inspiration was made from residual volume at maximal speed. The period of breath-holding was timed with a stopwatch and could be checked in retrospect from the tracing obtained at a paper speed of 3.2 cm per second. Ordinarily, the stopwatch was started at the beginning of fast inspiration and stopped at the beginning of expiration. Timing errors were never more than 1 second except in patients with severe emphysema when timing was always a problem. After 10 seconds the gas was exhaled rapidly. Exhalation of about 1 L was allowed for valve and lung dead space washout prior to alveolar sample collection, except in patients with a vital capacity of less than 1.4 L in whom 600 ml sufficed. A minimum of 350 ml was required for CO_2, CO and He analysis. The alveolar sample was then collected in a 2 L anesthesia bag with nipple.

Inspired and expired gases were analyzed for CO with an infrared meter.[3] With the low amplification factor used for these experiments, samples could be analyzed with a reproducibility such that the standard deviation in any large number of CO analyses was less than 1 per cent of the mean value. The zero reading was adjusted with dry room air and the full-scale reading with inspired gas. Although the meter is known to respond slightly to CO_2 due to overlapping absorption bands, gases containing up to 8 per cent CO_2 produced no detectable deflection with the amplification used. The meter reading for expired gas was converted to percentage of inspired gas by reference to our calibration curve. Details of measurement, calibration and over-all analytical error have been described elsewhere (8).

Helium concentrations were measured with a conventional catharometer. This instrument, however, responded significantly to CO_2 concentrations of the magnitude encountered: 5 per cent CO_2 caused an underestimation of the true helium concentration by about 0.8 per cent helium. Therefore, the gas sample always was first analyzed for CO_2 with another infrared meter,[4] or Scholander apparatus. Calibration curves constructed from known He-CO_2 mixtures, showing deflections due to varying amounts of CO_2 at given meter readings, were used for correction. The same results were obtained when the CO_2 was absorbed prior to helium analysis and a correction was made for volume change. Helium analyses were considered accurate within 0.2 vol per cent; an error of this magnitude may change the D_L by 2 to 3 per cent. It is evident from Equations 1 and 2 that a constant analytical error of either CO or He cancels if it is of the same magnitude for both inspired and expired gases; and that the use of helium as a tracer eliminates errors due to apparatus dead space of either the inspiratory or expiratory circuits. All gas volumes were corrected to STPD.

For measurements of the D_L at different lung volumes serial determinations were made. A long pointer, visible to the patient, was attached to the spirometer counterweight; the spirographic paper was marked at 1-L intervals and subjects were instructed to inspire to a different mark for each test. The actual volume inhaled on each occasion was measured from the spirogram with an accuracy of $\pm$ 10 ml. Exercise studies of both single breath and steady state D_L were made with subjects walking on a treadmill at 3 miles per hour on an 8 per cent grade after a steady state had been achieved.

The residual volume, required for calculation of V_A, was measured by the open-circuit nitrogen washout method (9), modified by addition of an alveolar trap for determination of the "pulmonary mixing index" and a box-balloon system to ascertain the "switching error" (10).

Carboxyhemoglobin (COHb) saturations, required for studies of back pressure, were determined according to the method of Gaensler and associates (8) in which all blood gases are extracted on the Van Slyke apparatus and analyzed with the infrared CO meter; this method is accurate to 0.02 vol per cent. The alveolar sampling method of Jones, Ellicott, Cadigan and Gaensler (11), in which the lungs are used as a tonometer, was used for rapid screening of COHb saturation and for determination of intermediate points during multiple studies.

The steady state $D_{L_{CO}}$ was measured by the "physiological dead space" method of Filley, MacIntosh and Wright (12) with the subject in the supine position. D_L may increase slightly with recumbency (13); the numerical differences here reported between the steady

[2] Box-balloon respirometer (no. P-1100) with five-way Hans Rudolph Valve (no. P-326). Warren E. Collins, Inc., Boston, Mass.

[3] Gas analyzer, model 15, Liston-Becker Co., Stamford, Conn.

[4] Gas analyzer, model 16, Liston-Becker Co., Stamford, Conn.

TABLE I

Variability of alveolar volume (V_A) and the reproducibility of the single breath diffusing capacity (D_{LCO})

Group	No. of observations	Alveolar volume (V_A)		Single breath D_L	
		Mean	Coefficient of variation	Mean	Coefficient of variation
		ml	%	*ml/min/mm Hg*	%
1 Normal woman (V_A not controlled)	20	3,781	±11.9	20.2	±12.9
12 Normal subjects (V_A not controlled)	37	4,870	± 7.2	30.6	± 8.9
26 Patients with alveolar-capillary block syndrome (1955–1956) (V_A not controlled)	52	3,770	± 5.7	16.8	±10.7
21 Patients with cardiac disease (V_A not controlled)	42	3,481	± 4.3	20.4	± 7.4
1 Normal man: V_A held "constant"	8	5,116	± 1.4	28.1	± 3.2
67 Patients with alveolar-capillary block syndrome (1957–1959)	221	Only V_A between 95–100% of best effort used		14.9	± 2.6

state and single breath methods, therefore, might have been even larger if the subjects had been in the same position during both determinations.

In the ensuing Discussion and illustrations, V_A at which the breath was held is expressed as per cent of the total lung capacity (TLC), defined here as the sum of the residual volume and the largest vital capacity obtained either by single breath maneuver or conventional spirography. Studies at a V_A lower than 40 per cent of TLC were difficult because about 1,350 ml of gas was required for dead space washout and alveolar sample. Further, in older individuals the residual volume itself often occupied as much as 40 per cent of the TLC.

RESULTS

1. Variation of D_L with alveolar volume. With clinical use of the single breath technique, large differences were frequently observed between duplicate measurements. For example, in 1955 and 1956 paired observations of 26 patients with the "alveolar-capillary block" syndrome resulted in a coefficient of variation for the D_L of 10.7 per cent; individual measurements varied one from another by as much as 39 per cent. The error appeared to be the same or greater in normal subjects and was somewhat smaller in cardiac patients with restrictive ventilatory insufficiency (Table I). Whenever large variations between duplicates were obtained, it was observed that the greater value was almost always associated with the deeper inspiration or larger alveolar volume, V_A. Since mere instruction to inhale maximally without practice or coaching resulted in variations of V_A by as much as 40 per cent, an effort was made to keep V_A constant. One normal subject, by observing the spirographic tracing, controlled inspiration so that for eight measurements of D_L, V_A remained within 70 ml of the mean. The coefficient of variation of the D_L for that series was reduced to 3 per cent (Table I). Because of this, during the next 3 years the lung volume has been considered in all clinical and statistical evaluations of single breath D_L. As a result the coefficient of variation has been markedly reduced. For example, in 67 recent patients with alveolar-capillary block, the coefficient of variation was 2.6 per cent compared with 10.7 per cent in similar cases studied before 1957 (Table I). This variation is considerably lower than any previously reported (7, 14).

These observations suggested studies which would permit quantitation of the relationship between V_A and D_L. A total of 248 determinations was made on 14 normal subjects and 15 patients with nonobstructive pulmonary disease (Tables II and III). The D_L was measured in each individual at 4 to 15 different V_A levels. The order of the latter was randomized to eliminate a systematic error due to accumulation of CO in the blood.

The results for normal subjects are summarized

TABLE II

Relationship of single breath D_{LCO} to lung volume and to steady state D_{LCO} in 14 normal subjects

Normal subjects	Age	Sex	Ht	Wt	Resid. vol (STPD)	Steady state D_{LCO}	Single breath D_{LCO} V_A range as per cent of maximal V_A						Lung volume (V_A)*			Single breath D_{LCO}*		
							100–90	89–80	79–70	69–60	59–50	49–30	Lowest	Highest (TLC)	Increase	Lowest	Highest	Increase
			cm	*kg*	*L*	*ml*	*ml/min/mm Hg*			*ml/min/mm Hg*			*L*	*L*	%	*ml/min/mm Hg*		%
E.H.†	28	M	171	78	0.76	17.0 (1)‡	31.0 (4)		26.9 (3)	22.4 (2)			3.27	5.36	+ 64	21.5	35.6	+66
A.M.†	29	M	186	81	1.12	24.5 (1)	34.2 (3)	25.7 (1)	26.3 (4)	25.0 (2)	25.9 (3)	24.5 (1)	2.24	5.64	+152	24.7	35.2	+43
J.A.†	35	M	178	77	1.26	15.6 (1)	27.3 (2)	23.2 (1)		18.2 (2)			2.68	4.41	+ 65	18.2	28.8	+58
B.F.	26	F	172	63	1.08	18.7 (2)	22.9 (3)	21.9 (4)		18.4 (4)	19.7 (1)	17.8 (1)	2.26	4.72	+109	17.8	23.2	+30
E.G.	35	M	183	81	1.41	16.0 (1)	33.2 (3)	28.6 (1)		27.3 (2)	26.6 (1)	24.5 (2)	2.48	5.83	+135	23.0	39.7	+73
H.K.	33	M	170	68	0.96		28.3 (5)	24.5 (1)	23.2 (3)	23.4 (1)			2.35	3.53	+ 50	23.4	29.6	+27
S.W.	24	M	179	71	1.46	21.2 (2)	36.8 (5)	30.3 (2)	28.1 (1)		29.7 (3)		2.97	5.41	+ 82	30.7	38.7	+26
M.S.	28	F	164	61	1.12	21.5 (2)	24.7 (9)	23.5 (2)	23.3 (2)	25.5 (1)	23.5 (1)	22.1 (1)	2.11	4.28	+103	22.1	27.4	+24
M.E.	30	F	161	52	1.04		20.6 (5)	15.7 (1)	11.0 (1)	12.7 (1)	14.6 (1)		2.16	3.75	+ 74	14.6	20.8	+42
L.H.	20	F	163	66	1.10		31.5 (3)	31.6 (2)		28.6 (2)	28.3 (2)		2.47	4.28	+ 73	26.4	31.1	+18
R.J.	31	M	188	91	1.58		34.6 (5)	35.1 (2)	31.8 (3)		29.3 (1)	26.0 (1)	2.91	6.02	+107	26.0	36.8	+42
J.C.	29	M	179	79	1.08		36.8 (2)	32.6 (3)	27.2 (1)	26.2 (3)	23.5 (2)		2.73	4.73	+ 73	23.2	34.1	+47
A.N.	22	F	169	65	0.84	17.3 (1)	21.2 (6)	19.4 (1)	20.3 (2)			13.7 (1)	2.01	4.12	+105	13.7	23.8	+74
F.S.†	32	M	174	72	1.63	18.0 (1)	36.4 (5)	31.4 (1)	29.6 (2)		23.4 (3)		2.54	4.96	+ 95	22.8	41.8	+83
Mean	28				1.17	18.9	30.0	26.4	24.8				2.51	4.79	+ 92	21.9	31.9	+47

* These data were calculated from lowest and highest V_A obtained in each subject.
† Data illustrated in Figure 1.
‡ Number in parentheses is number of determinations.

in Table II. Without exception there was a fall of DL as VA decreased. An average increase of VA of 91.9 per cent was accompanied by an average increase in DL of 46.6 per cent, and the latter increase was over 70 per cent in three subjects. Plotting of DL versus VA as percentage of TLC revealed three general types of curves, samples of which are shown in Figure 1. In some subjects the DL fell linearly with VA (E.W.H. and J.A.); in others there was an abrupt fall until VA reached 80 to 90 per cent of TLC after which a plateau was reached (A.M.). Decreases of this type were minimized in Table II because the data were grouped. A third curve, exemplified by Subject F.S., fell gently throughout the entire range.

Most of the patients shown in Table III had impaired diffusing capacities and classical alveolar-capillary block syndrome (4). For a given range of VA the mean DL values were lower than those of normal subjects, but for a smaller rise of VA there was an even larger rise of DL. This averaged 60 per cent for a 72 per cent rise of VA. Curves for four patients are shown in Figure 2.

During exercise the single breath DL increased as did the steady state DL, but even here a rise could be demonstrated with increasing lung volume (Table IV). In four subjects the mean increase of DL from minimal to maximal VA was 25 per cent both during rest and exercise.

A change of single breath diffusing capacity with changing lung volume might be anticipated from anatomic considerations. Because recent investigators have failed to demonstrate such variation (3, 6, 7) we recalculated the original data of Krogh (2) for comparison with present results. In two of her cases there was sufficient variation in

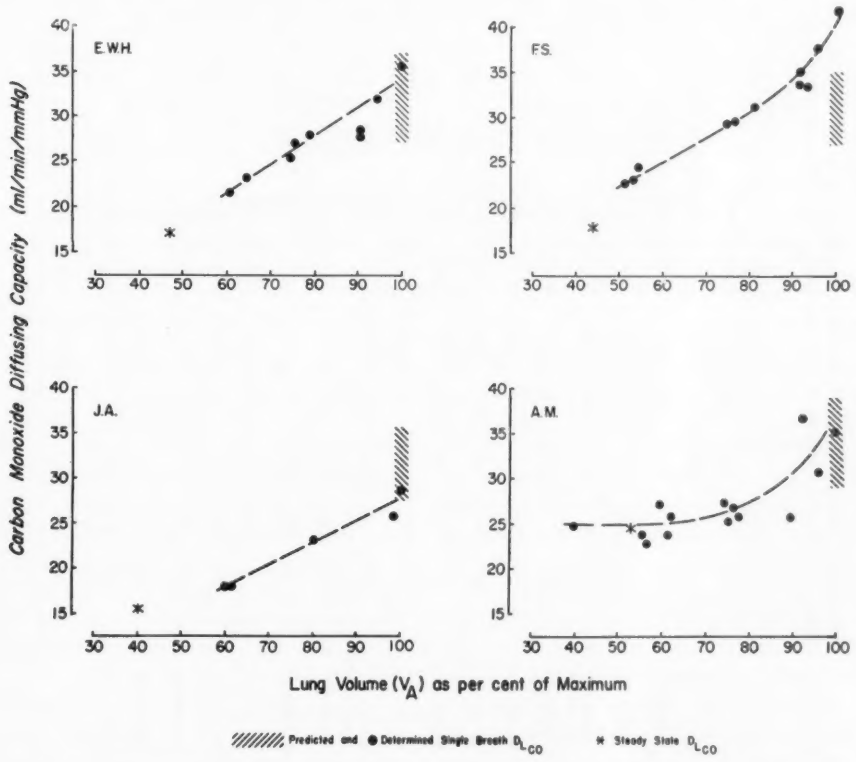

FIG. 1. THE EFFECT OF THE GAS VOLUME WITHIN THE LUNGS (VA) AT WHICH THE BREATH WAS HELD UPON THE SINGLE BREATH DIFFUSING CAPACITY (DL) IN FOUR NORMAL SUBJECTS (MEAN VALUES FOR GREATEST AND SMALLEST VA FOR 98 NORMAL SUBJECTS ARE SHOWN IN FIGURE 7). The stars indicate the physiological dead space steady state D_{LCO} (12); for this the effective VA was assumed to be functional residual capacity plus one-half tidal volume.

TABLE III

Relationship of single breath D_{LCO} to lung volume and to steady state D_{LCO} in 15 patients with pulmonary disease

Name	Diagnosis	Age	Sex	Ht	Wt	Resid. vol (STPD)	Steady state D_{LCO}	Single breath D_{LCO} V_A range as per cent of maximal V_A					Lung volume (V_A)*			Single breath D_{LCO}*		
								100–90	89–80	79–70	69–60	59–30	Lowest	Highest (TLC)	Increase	Lowest	Highest	Increase
				cm	*kg*	*L*	*ml*	*ml/min/mm Hg*			*ml/min/mm Hg*		*L*	*L*	*%*	*ml/min/mm Hg*		*%*
D.K.	Berylliosis	34	F	159	49	0.72		20.6 (3)†	17.6 (3)		16.7 (1)		2.40	3.44	+ 43	16.7	20.0	+ 20
P.K.	Berylliosis‡	39	M	178	81	0.72	8.6	21.8 (3)	16.8 (1)		14.3 (1)	12.4 (2)	1.68	3.84	+128	11.8	23.3	+ 97
L.P.	Pneumonectomy, tuberculosis	62	F	176	81	0.90		18.5 (2)	15.1 (5)	15.1 (1)	11.3 (1)		1.61	2.24	+ 39	11.3	19.9	+ 76
L.R.	Asthma, chr. bronchitis	40	F	168	66	1.39	14.4	20.7 (4)	20.0 (1)		17.6 (1)		2.27	3.44	+ 52	17.6	21.2	+ 20
R.T.	Sarcoidosis‡	27	M	168	61	1.04	15.2	25.9 (1)	21.9 (2)	18.3 (1)		16.1 (1)	1.94	4.36	+125	16.1	25.9	+ 61
E.W.	Berylliosis	31	F	169	74	1.00	6.3	17.1 (4)	14.8 (1)	13.1 (2)			2.26	3.03	+ 34	13.2	17.0	+ 29
C.H.	Berylliosis	36	M	180	85	0.70	8.4	22.6 (2)	18.9 (1)		15.3 (1)		2.64	4.07	+ 54	15.3	23.3	+ 52
W.G.	Berylliosis	48	M	171	68	0.57	4.2	6.7 (4)			3.1 (1)	2.0 (1)	1.47	3.00	+104	2.0	6.0	+200
E.D.	Miliary tuberculosis	36	M	172	100	0.94		31.4 (2)	29.3 (3)	23.3 (1)	24.2 (1)	23.6 (2)	1.70	3.14	+ 85	23.9	32.5	+ 36
V.DeA.	Berylliosis	27	M	179	82	1.57	14.1	32.5 (3)	28.0 (1)			23.7 (1)	2.83	4.90	+ 73	23.7	32.8	+ 38
E.M.	Silicosis	61	M	170	74	1.32		24.2 (2)	18.5 (1)		17.9 (1)		2.52	3.95	+ 57	17.9	23.1	+ 29
F.E.	Silicosis‡ Pre-Rx	55	M	170	70	0.71	13.6	18.7 (4)			14.5 (1)	11.9 (1)	1.81	3.11	+ 72	11.9	19.0	+ 60
F.E.	Silicosis‡ Post-Rx	55	M	170	75	0.86	11.4	19.7 (3)		16.4 (1)	14.9 (1)	12.6 (2)	1.69	3.28	+ 94	11.8	20.4	+ 73
F.D.	Pneumoconiosis	59	M	165	50	1.74	11.3	16.9 (2)				11.2 (2)	2.70	4.69	+ 74	11.4	17.3	+ 52
A.W.	Alv. proteinosis	16	F	168	59	0.43	15.1	18.1 (3)			15.0 (1)	12.9 (3)	1.29	2.84	+120	11.3	18.2	+ 61
H.C.	Pneumonectomy‡	33	F	167	57	0.52	9.5	16.5 (2)	13.8 (2)	11.3 (2)			1.28	2.00	+ 56	10.5	17.0	+ 62
Mean		42				0.94	11.0	20.7					2.01	3.46	+ 72	14.2	21.1	+ 60

* These data were calculated from lowest and highest V_A obtained in each subject.
† Number in parentheses is the number of determinations.
‡ Data illustrated in Figure 2.

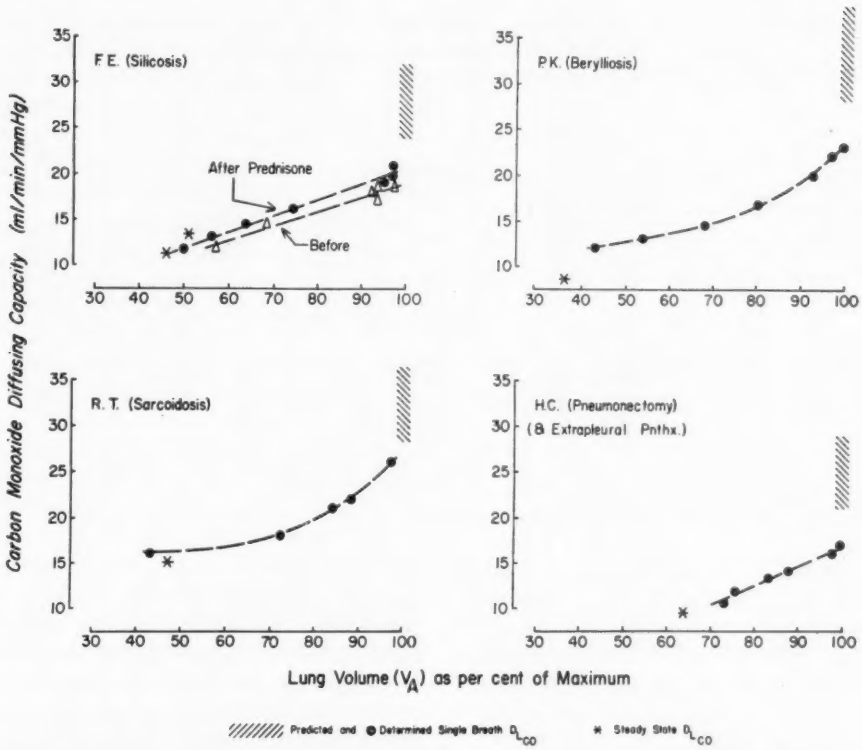

FIG. 2. THE RELATIONSHIP OF SINGLE BREATH DL TO VA PRESENTED AS IN FIGURE 1, BUT FOR PATIENTS WITH REDUCED DIFFUSING CAPACITY. The first three had an alveolar-capillary block syndrome, while the last (H.C.) had a pneumonectomy on one side and an upper extrapleural pneumothorax of the remaining lung.

VA so that a plot could be made (Figure 3). The similarity of these curves to our plateau type shown in Figure 1 requires no comment.

2. "Instantaneous DL." The demonstration that DL changes with VA, together with the possibility that DL may actually diminish during the period of breath-holding (3), suggests that the final value calculated by this method may be merely a composite due to several dynamic events occurring during the maneuver. An experiment was designed to measure the instantaneous value of DL *while* the VA increased during inspiration, thus eliminating the decrease of DL which may occur while the breath is held.

Spirometer paper was marked with slopes representing given rates of inspiration. After some training the subject was able to inspire at a constant rate following these pre-drawn lines. Only two comparatively slow rates of inspiration, 300 and 525 ml per second, were used because technical difficulties prevented inspiration at faster constant rates. Inhalation proceeded, starting from residual volume, at the predetermined rate and an alveolar sample was then delivered quickly without breath-holding. A number of points was obtained by ending inspirations at varying inspired volumes.

The rate at which $F_{A_{CO}}$ should have risen during inspiration, were it not for diffusion, could be calculated from the ratio of expired to inspired helium (Figure 4, curve A). The actual $F_{A_{CO}}$ obtained by gas analysis is indicated by curve B. The concentration of CO within the lungs at any moment is the quantity of CO which has been inspired less the CO which has left by diffusion. The volume of CO which has been taken up by the blood at any given moment can be calculated by subtraction: curve A − curve B = curve C.

TABLE IV

The effect of lung volume and exercise upon D_{LCO}*

Subject	Single breath D_{LCO} V_A range as per cent of maximal V_A									Steady state D_{LCO}			Per cent rise of single breath D_L from lowest to highest value	
	100–90			89–70			69–50							
	Rest	Exercise	% Rise	Rest	Exercise	% Rise	Rest	Exercise	% Rise	Rest	Exercise	% Rise	Rest	Exercise
M.S.	24.7 (9)†	34.2 (3)	+38	23.4 (4)	29.2 (1)	+25	24.5 (2)	29.6 (1)	+21	21.5 (2)	28.7 (1)	+ 33	+ 1	+16
S.W.	36.8 (5)	42.5 (2)	+15	29.5 (3)			29.7 (3)	31.5 (2)	+ 6	21.2 (2)	36.5 (1)	+ 72	+24	+35
A.M.	34.2 (3)	45.9 (2)	+34	26.1 (5)	39.5 (1)	+51	25.4 (5)			24.5 (1)	35.2 (1)	+ 44	+35	+16
E.H.	31.0 (4)			26.9 (3)	46.0 (1)	+71	22.4 (2)	35.0 (1)	+56	17.0 (1)	35.0 (1)	+106	+38	+31
Mean‡	31.9	40.9	+29	25.5	38.2	+49	25.5	32.0	+28	21.1	33.9	+ 64	+25	+25

* Steady exercise on treadmill on 8 per cent grade walking 3 miles per hour.
† Number in parentheses is number of observations.
‡ Means include only those values where both rest and exercise data were obtained.

The rate of CO loss due to diffusion at time t is the slope of curve C, dV_{CO}/dt. The "instantaneous" D_L (shown by curve D) can then be calculated for any given moment by dividing the slope of curve C (dV_{CO}/dt) by the partial pressure of CO in the alveoli (curve $B \times [B - 47]$) at that moment. The slope of curve C can be obtained graphically. The final curve, D, shows the instantaneous D_L obtained in this manner at the two rates of inspiration. At the slower rate the D_L changed with V_A just as with the plateau-type curves previously shown (Figure 1); at the faster rate the D_L starts to rise almost at once and continues to rise throughout inspiration. The shape of curve D would be altered materially by correction for back pressure, to be discussed subsequently. Obviously, the relationships shown in Figure 4 may be expressed algebraically in the form of differential equations.[5] Unfortunately, the final equation for curve D contains the expression for the slope of curve B or C which must still be obtained by graphic construction.

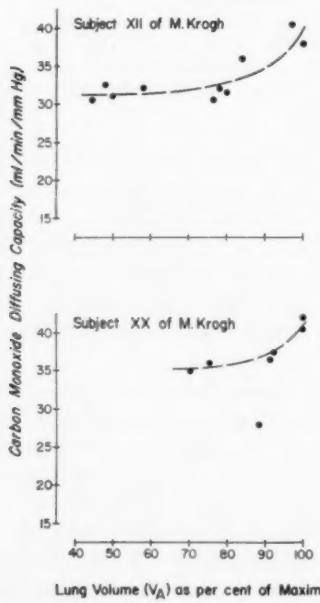

FIG. 3. THE RELATIONSHIP OF SINGLE BREATH D_L AND V_A IN TWO NORMAL SUBJECTS RECALCULATED FROM DATA OF KROGH (2).

3. The effect of CO back pressure upon D_L. The calculation of D_L by Equation 1 depends upon the validity of the assumption that the carbon monoxide in the pulmonary capillary blood exerts no significant pressure (back pressure). It is now known that there is not sufficient time in the pulmonary capillary for complete equilibration to occur between CO in free solution and that combined with hemoglobin (15, 16). The plasma CO tension in the capillary, which is neglected in Equation 1, is a complex function of the hemoglobin-carbon monoxide reaction time, mixed venous

[5] See Appendix.

carboxyhemoglobin saturation, rate of CO uptake, and the velocity of blood in the capillaries (1, 2, 15–17). Consequently, under the unique conditions of this test it may not be valid simply to use Haldane's "M" for the estimation of back pressure. To circumvent the problems of measuring the actual plasma tension, experiments were made to show: *a*) the change of COHb saturation with multiple single breath determinations, *b*) the effect of this change in saturation upon the DL actually obtained, and *c*) the order of magnitude of the back pressure which probably exists.

In five normal subjects the COHb saturation was raised by several periods of 0.1 per cent CO breathing. Multiple determinations of DL (at maximal VA) and of COHb saturations were made before and after each CO breathing period. At least 5 minutes was allowed between each test and each CO breathing to permit re-equilibration of the lungs with ambient air. The CO uptake resulting from both the CO breathing and the breath-holding tests was calculated and recorded. The measured COHb saturation was compared to a COHb saturation calculated from this CO uptake and an assumed blood volume. The two values were nearly the same at saturations up to 15 per cent. At higher saturations the measured blood saturation was always lower than that calculated from CO uptake, presumably because of the greater rate of CO loss from the lungs during intervals between tests.

The single breath DL obtained at constant VA was plotted for different COHb saturations (Figure 5). The slopes indicate the decrease in apparent DL with increasing COHb saturation. This decrease ranged from 0.15 to 0.32 ml per minute per mm Hg per 1 per cent COHb, with a mean value of 0.27. Extrapolation of the slopes to the ordinate was somewhat uncertain in the two smokers, E. G. and J. C., and not difficult in the three nonsmokers. If one assumes that the back pressure is zero at the ordinate where COHb saturation is zero, then the intercept would be the "true DL." Further, it should be possible to correct DL values observed at other than zero COHb saturation for the effect of back pressure. Such a correction was made for all values in each subject (Figure 5) using Haldane's equation, assuming M = 245, mean pulmonary capillary Po_2 = 85 mm Hg, and COHb + O_2Hb = 95 per cent

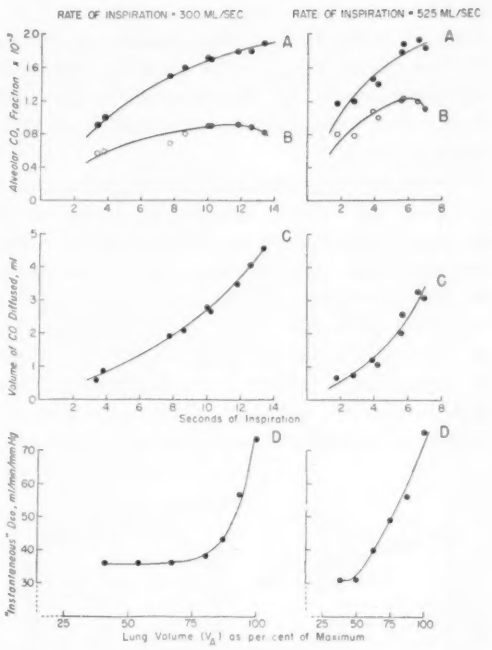

FIG. 4. THE INSTANTANEOUS DL OBTAINED WITHOUT BREATH-HOLDING DURING TWO DIFFERENT STEADY RATES OF INSPIRATION. The rate of increase of alveolar CO tension which would have existed if no diffusion had occurred (curve A) was calculated from helium ratios. The actual alveolar CO tension, obtained from alveolar samples, is indicated by curve B. The volume of CO which had diffused out of the lungs at any given moment (curve C) was obtained by subtracting curve B from curve A. The rate at which CO left the lungs, indicated by the slope of curve C, was obtained graphically. This slope, at any point, divided by the alveolar CO tension at that time is, by definition, the diffusing capacity. This instantaneous DL plotted against the lung volume (VA) at that instant is indicated by curve D. (Note the change in abscissa.)

(11). The corrected values should lie on a horizontal line at the level of the true DL. However, this was never the case since the back pressure correction, so calculated, was invariably too low. In fact, no adequate correction could be obtained with Haldane's equation using several other reasonable values for M, $\bar{P}a_{O_2}$, and saturation.

An "effective back pressure," P_e, was calculated for each observed DL value such that the corrected DL became equal to true DL, again assuming that back pressure is zero at zero COHb saturation. These P_e values, plotted against corresponding

COHb saturations at the bottom of Figure 5, resulted in carboxyhemoglobin dissociation curves effective during these special conditions for each individual subject. P_e was from 1.3 to 2.5 times greater than back pressures calculated from Haldane's equation.

Forster and co-workers (3) found that, contrary to theory, the relation between the logarithm of alveolar CO tension and the time of breath-holding was not linear even when correction was made for back pressure. Three such breath-holding curves are plotted for E. G. at 5.5, 11.8 and 17.0 per cent COHb saturation in Figure 6. If the P_e values from Figure 5 are subtracted, three straight lines are inscribed. The empirically calculated P_e correction from Figure 5 must produce the same slope for 10-second breath-holding at all COHb saturations, since this was the assumption for calculation of P_e. The fact that the corrected slopes are the same also at 20 seconds, despite differences in COHb saturation, and that straight lines are produced up to 60 seconds in the two other subjects (Figure 6) suggests that the observed alinearity is not the result of different diffusing phases, as stipulated by Forster and associates (3) but rather due to back pressure. Decay curves at two different lung volumes in Subject A. M. (Figure 6) were also straightened but the slopes remained dissimilar. This suggests that the change in DL with VA is not due to back pressure effect alone. These results appear to support the original assumption that back pressure is zero at zero COHb saturation, that the concept of true DL is valid, and that P_e, in fact, is the effective back pressure.

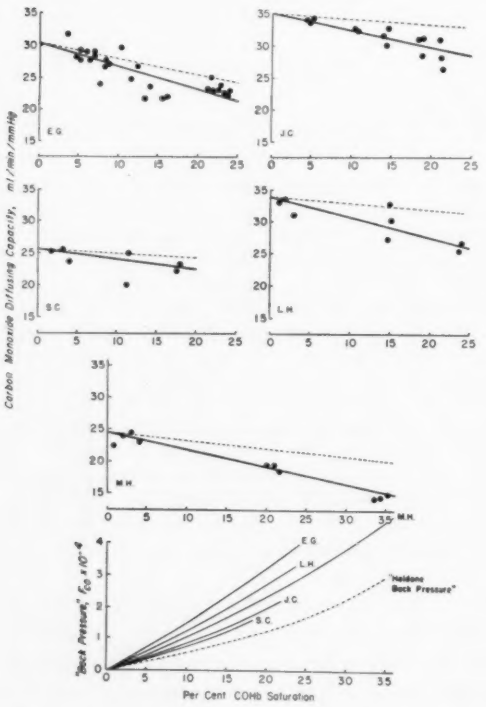

FIG. 5. ALTERATION OF THE APPARENT DL WITH INCREASING COHb SATURATION. The first two subjects (E. G. and J. C.) were smokers; the other three were not. The broken lines indicate the "correction" of DL resulting from use of Haldane's equation. The lowest graph shows the effective back pressure at various COHb saturations assuming: *a*) a zero plasma CO tension at zero saturation, and *b*) that the progressive decline of DL was solely a function of back pressure.

4. The relationship between the steady state and single breath methods. The steady state $D_{L_{CO}}$ and $D_{L_{O_2}}$ values are usually quite comparable, but the single breath $D_{L_{CO}}$ is always much larger. For example, in nine normal subjects the mean steady state value at rest was 18.9 ml per minute per mm Hg compared with 32.7 ml by the single breath technique (Table II), and in patients without obstructive disease the latter value was 2.4 times larger than the former (4). However, if the two determinations are made at comparable lung volumes (considering VA for the steady state maneuver to be equal to FRC plus one-half the tidal volume), the difference vanishes. In five of our normal subjects (Table II) we obtained the DL by both techniques at comparable VA, that is, 30 to 49 per cent of TLC. For these five, the steady state DL averaged 19.6 and the single breath DL at comparable VA averaged 20.5 ml per minute per mm Hg. The mean single breath DL for the same five subjects at maximal inspiration (90 to 100 per cent of TLC) was 27.2. The same was true for the eight patients of Table III in whom values were obtained at comparable lung volumes; here the mean single breath DL (at 30 to 59 per cent of VA) was 12.8 and the mean steady state DL 11.8 ml per minute per mm Hg. This relationship is also indicated in Figures 1 and 2.

It seemed, therefore, that the different results obtained by the two techniques were a function of

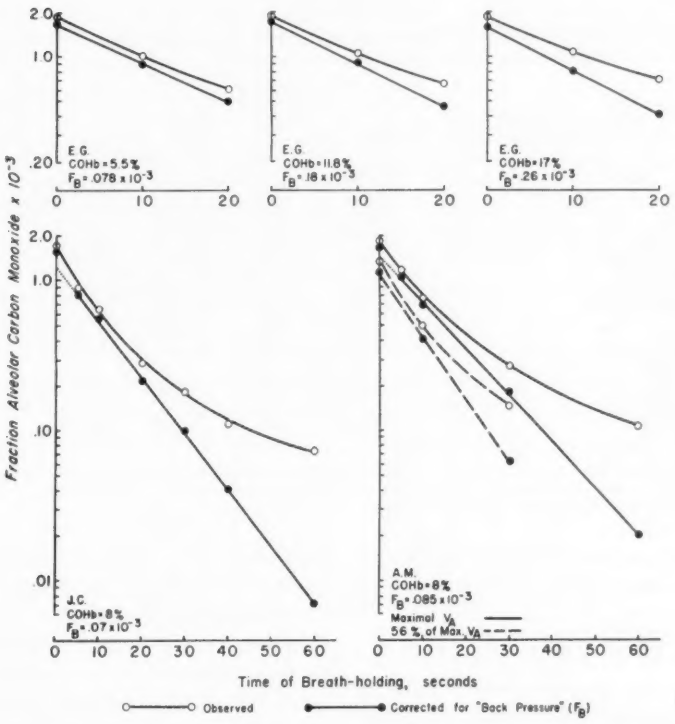

FIG. 6. THE RELATIONSHIP OF THE NATURAL LOGARITHM OF ALVEOLAR CO CONCENTRATION TO THE TIME OF BREATH-HOLDING WITHOUT CORRECTION FOR BACK PRESSURE AND WITH CORRECTIONS OBTAINED FROM DATA OF FIGURE 5. All values were obtained at maximal VA except the second set of Subject A. M. for which the breath was held at 56 per cent of VA.

the lung volume at which the measurements were made. Attempting to prove this, experiments were performed in which lung volume was changed during steady state CO breathing. Three subjects were placed in a body plethysmograph with head and one arm protruding for arterial sampling. VA was changed by altering the pressure within the chamber stepwise from + 10 to − 20 cm of water. A 20 cm decrease in pressure resulted in an increase in FRC of 1,200 to 3,000 ml. At least 30 minutes was allowed between each determination for return to a steady state at the new FRC. All measurements on a single subject were made within a 5 hour period. The data are arranged in Table V according to increasing VA. Under these experimental conditions, no increase of DL could be demonstrated with an increase in lung volume. On the contrary, there seemed to be a slight decrease which was presumed to have resulted from a progressive rise of COHb saturation and hence a rise in back pressure. This is emphasized if tests are rearranged in order of performance instead of lung volume. To correct for this factor, the plasma tension was estimated from Haldane's equations and known CO uptake. This correction failed to reveal a rise of DL with VA, and examination of Table V suggests that no manner of back pressure correction could alter the data to show a concomitant rise of DL with VA. The results are further complicated by the fact that lowering of plethysmograph pressure has the same physiological consequences as continuous positive pressure breathing and its effect on thoracic, and possibly on pulmonary capillary, blood volume. Since these studies were made, Grape and Tyler (18), using end tidal sampling measurements, were able to demonstrate some increase of steady state DL with increasing FRC, but since then we have not

TABLE V

The steady state D_{LCO} with lung volume variations induced by altering ambient pressure

Subject	Order of expts.	Body tank pressure	Minutes of 0.1% CO breathing	CO uptake	$\bar{P}_{ACO}$	Estimated* % COHb during 3-min test	Estimated CO back pressure	D_{LCO}	D_{LCO} corrected for back pressure	Lung volume (V_A)
		cm H_2O		*ml/min*	*mm Hg*		*mm Hg*	*ml/min/mm*		*ml*
E.G.	1	0	10	2.35	0.144	6.45	0.028	16.3	20.2	1,698
	3	−10	10	2.22	0.166	8.85	0.040	13.4	17.6	2,704
	2	−15	12	2.12	0.146	7.85	0.035	14.5	19.1	3,340
	4	−20	9	2.17	0.186	9.95	0.046	11.7	15.5	4,183
W.A.	3	+10	9	1.82	0.154	8.18	0.035	12.6	15.3	1,631
	1	0	9	2.26	0.146	6.27	0.028	15.5	19.2	2,186
	2	−10	9	2.00	0.142	7.27	0.032	14.1	18.2	3,210
	4	−20	9	2.12	0.150	8.98	0.041	14.1	19.4	4,567
H.M.	3	0	9	5.33	0.258	8.18	0.035	20.7	23.9	2,093
	2	−10	9	5.49	0.256	7.27	0.032	21.4	24.5	2,573
	1	−20	9	5.75	0.268	6.27	0.028	21.5	24.0	3,204

* Initial COHb saturation was estimated from smoking history; for each minute of 0.1% CO breathing a 0.17% rise in COHb saturation was assumed; the derived value is the mean for the last 3 minutes of the first test. Subsequent tests were made at 30-minute intervals; for these a half-life COHb of 4.5 hours was assumed.

been able to reproduce their results either by the physiological dead space technique or by end tidal sampling.

DISCUSSION

Analysis of previous data from our laboratory was made, and a series of experiments was undertaken, in an attempt to explain the considerable variability of the single breath D_{LCO}, and its large numerical difference from the steady state values.

Variations in the lung volume at which the breath was held appeared to explain in large part the variability of the test (Table I). Careful control of the lung volume has increased the clinical usefulness of the procedure (5). A rising back pressure was ruled out as a source of serious variation in repetitive tests (Figure 5). The cumulative effect of plasma CO was so small that the progressive change in D_L was within the limit of error of the various physical analytical procedures unless more than 10 or 12 consecutive observations were made.

As soon as the test was devised by Krogh (2) she noted changes in the diffusing capacity related to alveolar volume. Because D_L decreased in proportion to V_A until FRC was reached but remained unchanged thereafter, she postulated that the effective membrane progressively decreased in size down to FRC, but that further reduction of D_L from this point on was prevented by infolding of the membrane. Forster, Ogilvie and their associates (3, 6, 7, 19) were not able to confirm this finding and thought that Krogh's results were due to the variable conditions of the initial alveolar sampling which they had eliminated by the use of helium. Recently, they reported data of five patients with a mean increase of D_L of only 9 per cent as V_A rose 53 per cent from 3 to 4.6 L (7). However, the low V_A was considerably above FRC, and the inclusion of one subject with an unusually high D_L at the low volume weighted the results. Excluding this individual, the mean increase of V_A would have been 15 per cent.

Data obtained with the helium technique supporting Krogh's contention were first mentioned in a study from our laboratory comparing different methods (4), and have since been confirmed by others (14, 20, 21). More recently, in an effort to develop a prediction formula for the single breath D_L, we found a significant variation of D_L with V_A in all but 4 of 98 normal subjects (Figure 7); and in 22 patients with chronic beryllium poisoning who had a series of single breath tests at widely differing lung volumes, the expected correlation with V_A was found every time (5).

The second problem, the numerical difference between the apparent D_L determined by the single breath and the steady state techniques, appeared to be closely related. Unlike the controversy concerning the single breath D_L-V_A relationship, there is little argument that, at rest, the single

breath test results in much larger values than the steady state DL, however determined (4, 14, 20, 22). A single dissenting opinion (13) appears to have been based on a laboratory analytical error (23).

The total resistance to diffusion, 1/DL, has been shown to be a composite of the resistance across the pulmonary membrane separating alveolar air from the surface of the red cell, 1/DM, and the "intracapillary resistance" due to the time taken for CO diffusion into the red blood cells and reaction with hemoglobin, $1/\theta V_c$; θ is the number of milliliters of CO taken up by the red cells in 1 ml of blood per minute per 1 mm Hg pressure gradient of dissolved gas between the plasma and the interior of the red cell, and V_c is the total volume in milliliters of blood in the lung capillaries exposed to alveolar air (17, 24).

Consideration of the factors affecting the magnitude of DL obtained by different techniques and at different lung volumes thus must include: *a*) possible variations of DM and V_c, *b*) whether CO back pressure is equally "negligible" under all circumstances, and *c*) whether in a given individual "the same lung" is being sampled under different circumstances.

The reaction rate, θ, can be excluded from consideration. In the same individual at rest, θ can be changed only by altering significantly the mean pulmonary capillary oxygen tension. This is not the case in the techniques under consideration.

The diffusing capacity of the membrane, DM, using the same gas, can be altered only by changing the thickness or area or both of the gas-blood interphase. Krogh (2) and several investigators since (14, 20, 25, 26) have attributed the increase of DL with increasing VA to a stretching and consequent thinning and increase in area of the membrane. Even the increase of DL with exercise has been attributed to a larger membrane surface resulting from an elevation of the pulmonary midposition (25, 26). Few data appear to support this thesis. The fact that the steady state DL value falls on a line relating single breath DL and VA at a point where VA approximately equals FRC (Figures 1 and 2) may be fortuitous because the usual calculations underestimate the true steady state DL (27). Our determinations of this value during body-respirator-induced increase of FRC also do not lend support to the "stretch theory."

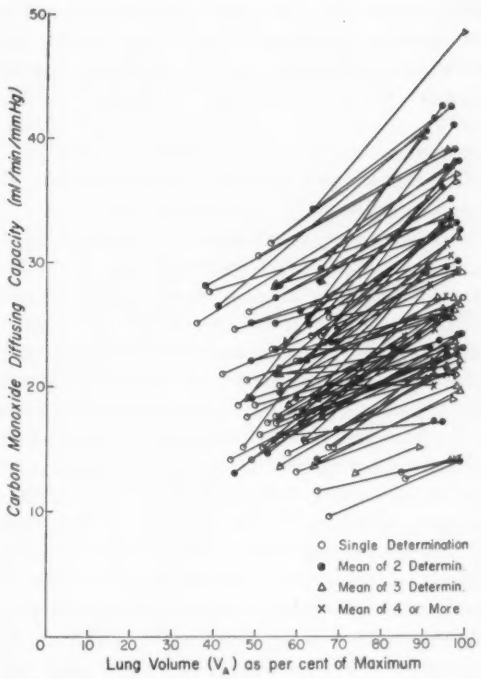

FIG. 7. THE RELATIONSHIP OF SINGLE BREATH DL AND VA IN 98 NORMAL SUBJECTS. Mean DL values are shown only for the highest and lowest VA achieved. Individual curves are shown in Figure 2.

Actually, decisive experiments in this regard have yet to be performed. These will have to include steady state DL measurements at various lung volumes *without alteration of airway pressure;* and single breath measurements *without initial Müller-like maneuver.*

Alteration of pulmonary capillary blood volume, V_c, may be the decisive factor causing both the changes of single breath DL with VA and the numerical difference from the steady state values The following evidence is cited: all manner of DL measures have been shown to increase during recumbency (13) and after central venous engorgement (28), both of which cause an increase of V_c; respiratory gymnastics, such as voluntary hyperventilation, cause an increase of steady state DL which may equal the increase seen during exercise requiring ventilation of comparable magnitude (22, 26, 29); positive pressure breathing, which decreases V_c, tends to decrease the steady state DL even though it causes a considerable increase

of V_A (Table V); and negative pressure breathing increases the steady state D_L (30). Finally, the instantaneous D_L varies with lung volume depending upon the speed and vigor of inspiration (Figure 4). Each of these observations suggests that D_L changes as the result of variations in V_c. This inferential evidence has been confirmed recently by direct estimates of V_c (30–32).

During normal inspiration the volume of blood returning to the heart increases and, for a few beats, the right ventricular ouput exceeds that of the left. Although this increase is largely due to extracapillary pulmonary and thoracic blood, it must reflect, in part, on V_c. A Müller maneuver has a similar effect. The considerable reduction in intrathoracic pressure incident to maximal inspiration from residual volume for performance of the breath-holding test should have a much more marked effect because, simultaneous with increase in lung volume, the capillary bed is free to expand. Indeed, in one normal subject we were able to show an almost linear relationship between peak negative intrathoracic (esophageal) pressure required for rapid inspiration of various lung volumes and the resulting D_L. The instantaneous D_L is another demonstration of this relationship. Furthermore, Roughton and Forster (17) measured and calculated separately D_L, D_M and V_c during steady state, 30-second and 10-second breath-holding in five subjects. As always, the 10-second breath-holding D_L was much larger (50 per cent) than the steady state D_L, but D_M was quite comparable by all three techniques. Interestingly, V_c averaged 58.9 ml for the steady state method, 66.2 ml for 30-second breath-holding, and 78.7 ml for 10-second breath-holding. This lends support to our data which suggest that the more violent and the more recent the preceding inspiration, the greater V_c and D_L. As breath-holding proceeds the glottis is closed and the chest is relaxed against V_A, or even an expiratory force may be exerted. This Valsalva-like maneuver decreases cardiac output and, presumably, V_c and D_L. Actually, measured increases of intrathoracic pressure of 37 to 64 mm Hg decreased D_L by a maximum of 17 per cent (7).

Back pressure. The relative error of each method due to ignoring of back pressure must also be considered. Our procedure and assumptions for estimating effective back pressure, P_e, during the breath-holding test appear to be valid because pressures so obtained corrected for the alinearity of the ln $F_{A_{CO}}$ versus time relationship under a variety of circumstances (Figure 6).

Data for eight normal subjects who had determinations of both single breath D_L (at 90 to 100 per cent of V_A) and of resting physiological dead space steady state D_L are shown in Table VI. The two uncorrected D_L values, averaged for several observations, are shown in columns b and h. The mean values for the whole group were 29.9 and 19.2 ml per minute per mm Hg, respectively. Initial carboxyhemoglobin saturations (column a) were estimated from smoking histories (8). The back pressures effective during the single breath test, P_e (column c), were taken from the curves of Figure 5, and averaged 0.030 mm Hg. This corrected the single breath D_L to a mean value

TABLE VI

The relative effects of back pressure upon single breath and steady state $D_{L_{CO}}$ *

				Single breath D_L					Steady state D_L						
Subject	Sex	No. of cig.	S_{CO}	No. of obs.	D_L	P_e	D_L corr.	Changes of D_L corr.	No. of obs.	$\bar{V}_{CO}$	$\bar{P}_{A_{CO}}$	D_L	D_L corr.	Changes of D_L corr.	P_e
			(a) %		(b)	(c)	(d)	(e) %		(f)	(g)	(h)	(i)	(j) %	(k)
S.W.	M	0	1.0	5	36.8	0.018	37.8	2.6	2	2.81	0.133	21.1	24.4	13.5	0.059
M.S.	F	0	1.0	9	24.7	0.018	24.8	0.4	2	1.85	0.086	21.5	27.2	20.9	0.011
E.H.	M	0	1.0	4	31.0	0.018	31.5	1.6	1	1.97	0.117	16.8	19.9	15.5	0.055
B.F.	F	<15	2.0	3	22.9	0.025	23.7	3.4	2	2.90	0.154	18.8	22.4	16.1	0.032
A.M.	M	30–40	6.0	3	34.2	0.053	37.7	9.3	2	3.93	0.159	24.7	37.1	33.5	0.055
J.K.	M	20–30	5.0	2	34.8	0.046	35.7	2.4	1	3.86	0.225	17.2	21.6	15.8	0.117
E.G.	M	20–30	5.0	3	33.2	0.046	34.9	4.9	1	2.35	0.144	16.3	24.0	39.6	0.064
A.N.	F	0	1.0	6	21.2	0.018	21.3	0.5	2	2.44	0.142	17.2	19.7	13.7	0.028
Mean			2.75		29.9	0.030	30.9	3.1		2.76	0.145	19.2	24.5	21.1	0.050

* (a) Carboxyhemoglobin saturation estimated from smoking histories (8). (b) Obtained at V_A between 90–100% of maximal V_A. (c) Obtained from Figure 5 (CO-Hb dissociation curves applying during breath-holding). (d) $\frac{V_A}{BP-47} \ln \left[\frac{P_{A_{CO_i}} - P_e}{P_{A_{CO_f}} - P_e} \right] \times \frac{1}{t}$. (e) $\frac{d - b}{d} \times 100$. (f) Mean CO uptake. (g) Mean alveolar CO partial pressure. (h) f/g. (i) f/g-c. (j) $[(i - h)/i] \times 100$. (k) Calculated from Equation 3.

of 30.9 ml per minute per mm Hg (column d), an increase of only 3.1 per cent (column e). Application of the same back pressure, P_e, to the steady state D_L (column h) increased this value to 24.5 ml per minute per mm Hg (column i), a considerably larger change of 21.1 per cent (column j). The effect of a back pressure correction of given magnitude on the steady state D_L is thus seven times greater than the effect of the same correction on the single breath D_L. Nevertheless, the corrected steady state D_L (column i) still remains considerably lower than the corrected single breath D_L (column d).

If a larger back pressure during the steady state procedure were the only cause for the lower value obtained, then the back pressure during the steady state test, P_s, could be calculated by assuming equality for the two D_L values, after correction of the single breath D_L for P_e:

$$D_L \text{ (single breath, corrected for } P_e) = \frac{\text{CO uptake (steady state)}}{\text{alveolar CO tension (steady state)} - P_s} \quad [3]$$

This equation was solved for P_s for the eight patients shown in Table VI (column k). The mean P_s value for the entire group was 0.050 mm Hg. Hence, if back pressure were the sole reason for the discrepant values obtained by the two methods, then the back pressure during the steady state test would have to be nearly twice as large as that prevailing during the single breath test. That this hypothetical P_s pressure is much too high, and that factors other than back pressure must be responsible for the numerical difference between the two tests, is suggested by Linderholm (27). He estimated back pressures obtaining during the steady state D_L from arterial blood CO analyses or from the CO uptake and an assumed total amount of hemoglobin. In nine subjects studied under the same conditions as ours, that is, at rest breathing 0.1 per cent CO, the mean initial carboxyhemoglobin saturation of 4.7 per cent was nearly twice as great as ours (Table VI), yet back pressures averaged only 0.022 and 0.014 mm Hg by the two methods of estimation.

From this we conclude: *a*) that the true back pressure for the steady state method must lie between P_e and P_s; *b*) that although the actual steady state D_L value is probably 20 to 40 per cent larger than the uncorrected value, it still does not equal the single breath D_L obtained at maximal V_A; and *c*) that back pressure alone contributes only in small part to the greater value of the single breath D_L.

Finally, it must be questioned whether *different techniques sample "the same lung"* in a given individual. Forster (19) reviewed the theoretical effects of non-uniformity of lung on estimation of pulmonary diffusing capacity. He concluded that the physiological dead space steady state technique, which is the only steady state method we have used, tends to give lower values for D_L than the single breath method, especially in the presence of non-uniform alveolar blood flow:ventilation ratios. Marshall (20) concluded on the basis of results of fractional alveolar gas sampling that steady state methods, particularly the alveolar sampling techniques, measure combined effect of poor diffusion and unequal ventilation while single breath measurements are independent of unevenness of ventilation and affected only by membrane and capillary blood volume. Therefore, he felt that the single breath technique is a measure of the potential diffusing capacity.

Perhaps there is an analogy between D_L and lung compliance (C_L) measurements in patients with markedly non-uniform ventilation. In these a very large C_L value is obtained during breath-holding, because the entire lung is sampled. With increasing frequency of breathing, compliance steadily decreases because there is more and more "trapping," and as a result a progressively smaller and smaller lung is being sampled (33). Indeed, failure of steady state D_L to increase with exercise in an occasional patient with severe emphysema may be explained on this basis alone.

If non-uniform ventilation were the sole cause of the numerical difference between steady state and single breath D_L measurements, then this difference should be: *a*) very small in normal subjects, *b*) greater in patients with lung disease, and *c*) roughly proportional to non-uniformity of lung ventilation as demonstrated by other techniques.

The first of the three suppositions is incorrect. The difference is considerable even in normal subjects (4), suggesting that factors other than non-uniform ventilation must play a role. The second supposition, that the numerical difference should be greater in patients with pulmonary dis-

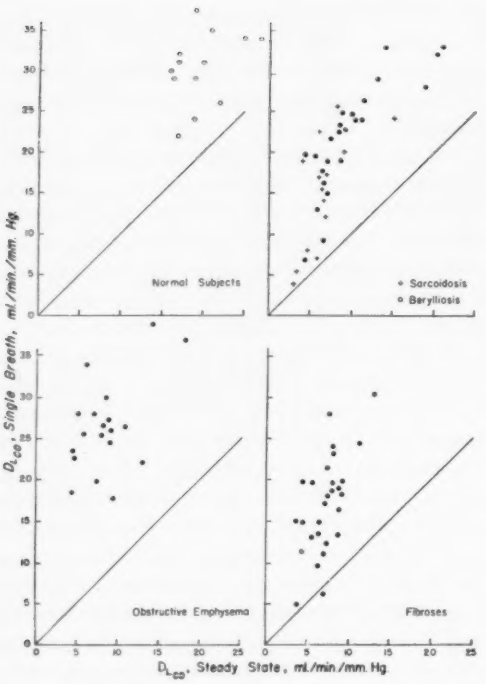

FIG. 8. THE RELATIONSHIP BETWEEN THE RESTING (SITTING) SINGLE BREATH DL OBTAINED AT MAXIMUM VA, AND THE RESTING (SUPINE) STEADY STATE PHYSIOLOGICAL DEAD SPACE DL IN 13 NORMAL SUBJECTS AND IN 85 PATIENTS WITH PULMONARY DISEASE.

ease than in normal subjects, is borne out by a large number of data from our laboratory. Figure 8 shows plots of single breath DL against physiological dead space steady state DL. The single breath value was larger and the discrepancy was greater in patients than in normal subjects, and was greatest in emphysema. In 13 normal young subjects in whom ventilation-perfusion ratios presumably were nearly "ideal," the single breath DL was 1.6 times larger than the steady state DL. In patients with relatively "pure" alveolar-capillary block there is usually unevenness of blood flow (4, 5) and occasionally uneven ventilation (34). In 28 such patients with diffuse fibroses, the single breath DL was 2.3 times larger than the steady state DL (4). In 14 patients with sarcoidosis and in 24 with berylliosis, including a few with complicating emphysema, it was 2.5 times larger (5). Finally, in 19 patients with moderate to far-advanced emphysema, the single breath DL was 3.4 times or almost 200 per cent larger (35). These relationships are shown in Figure 8.

In emphysema familiar landmarks such as "dead space," "alveolar gas" and "effective ventilation" virtually disappear (33). Therefore, several of the assumptions required for calculation of either $D_{L_{O_2}}$ or $D_{L_{CO}}$ are certainly not true and the required expression of $\overline{P}_{A_{CO}}$ becomes a rather nebulous value. Indeed, Briscoe and co-workers (36) have recently shown that in emphysema the ventilation:perfusion ratio may be ten times higher in well than in poorly ventilated alveoli with corresponding end-capillary oxygen saturations ranging from 97.5 to 76 per cent. They conclude that nonhomogeneity of this severity invalidates DL calculations based upon any single mean O_2 or CO tension, and alveolar ventilation based on equating alveolar P_{CO_2} with arterial P_{CO_2}. Because of such problems we have never previously reported diffusing capacity measurements in emphysema and, indeed, have always excluded all patients with grossly non-uniform alveolar ventilation from our discussions of diffusion impairment (4, 37). However, to test the third supposition, that the discrepancy between steady state and single breath DL measurements should be roughly proportional to non-uniformity of lung ventilation, a comparison was made in the upper half of Figure 9 of 13 normal subjects and of 19 patients with emphysema. The ratios of DL single breath : DL steady state were plotted against the pulmonary mixing indices or the percentage residual alveolar N_2 after 7 minutes' oxygen breathing (9), a moderately sensitive test of the non-uniformity of alveolar ventilation. A close relationship was readily demonstrated. In normal subjects and in patients with less than 2 per cent residual N_2, the single breath DL was at most 2 times larger than the steady state value; with residual N_2 from 2 to 6 per cent it was up to 3.5 times larger; and with severely uneven ventilation, usually in bullous emphysema, it was as much as 5 times larger. The data further show that patients with severest emphysema had the lowest steady state diffusing capacity; and that the lower this steady state DL, the greater the discrepancy between it and the single breath value, as demonstrated in the lower plot of Figure 9.

These observations suggest that inequalities of ventilation, of perfusion or of ventilation-perfusion

ratios, are important factors in the discrepancy between results of physiological dead space steady state and single breath DL methods. At least in some individuals the two tests do not sample "the same lung."

SUMMARY AND CONCLUSIONS

The helium dilution modification of the Krogh single breath technique is an attractive method for measurement of the apparent diffusing capacity of the lungs (DL) because it is bloodless and, with known residual volume, requires but a few minutes. However, experience with this method during the past 7 years has emphasized certain problems. These were: *a*) failure of repeated tests to check closely, *b*) a much larger numerical result than that obtained with O_2 or CO steady state DL techniques in normal subjects, and *c*) an even greater discrepancy in patients with pulmonary disease. The following observations were made in an attempt to clarify these problems.

Variations between multiple tests were found to be due to variations in the volume of the lungs (VA) at which the breath was held. In normal subjects the coefficient of variation was reduced from 8.9 to 3.2 per cent merely by holding VA constant; and in patients who took a "deepest possible breath" duplicate tests had a coefficient of variation of 10.7 per cent which was reduced to 2.6 per cent if VA was controlled at 95 to 100 per cent of total lung capacity.

A quantitative relationship between DL and VA was demonstrated. Four to 15 DL determinations were made at various lung volumes: in 14 normal subjects a 92 per cent increase of VA caused an average increase of DL of 47 per cent and of more than 70 per cent in 3 subjects; in 15 patients this increase averaged 60 per cent. Changes of similar magnitude were observed during exercise. Subsequently, with over 600 determinations in 98 normal subjects this DL-VA relationship failed to occur only four times.

Both in normal subjects and in patients, the single breath DL was invariably larger than the "physiological dead space" steady state DL.

The following variables were considered to explain these observations:

1. *The effective CO "back pressure"* did not measurably alter duplicate single breath determinations. However, in five normal subjects a 1 per cent increase of carboxyhemoglobin (COHb) saturation decreased the apparent DL by 0.15 to 0.32 ml per minute per mm Hg, and at 25 per cent COHb saturation the DL was, on the average, 6.8 ml per minute per mm Hg less than at zero COHb saturation. Effective back pressure was from 1.3 to 2.5 times greater than estimates obtained from Haldane's relationship. Although back pressure caused an underestimation of the average single breath DL of only 3.1 per cent and of the average steady state DL by 21.1 per cent, this did not fully explain the discrepant results. Back pressure did explain the previously observed alinearity in the relationship of the duration of breath-holding to the natural logarithm of alveolar CO concentration.

2. *An increase of the diffusing capacity of the*

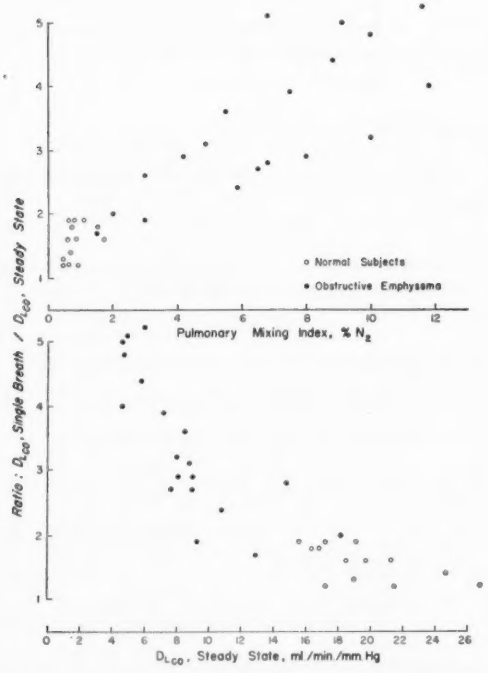
Ratio : $D_{L_{CO}}$ Single Breath / $D_{L_{CO}}$, Steady State
o Normal Subjects
• Obstructive Emphysema
Pulmonary Mixing Index, % N_2
$D_{L_{CO}}$, Steady State, ml./min./mm Hg

FIG. 9. THE RATIO OF RESTING SINGLE BREATH DL: STEADY STATE DL COMPARED WITH (TOP) THE ALVEOLAR NITROGEN CONCENTRATION AFTER 7 MINUTES' OXYGEN BREATHING (THE PULMONARY MIXING INDEX) AND (BOTTOM) WITH THE ACTUAL STEADY STATE DL, IN 13 NORMAL SUBJECTS AND IN 19 PATIENTS WITH MODERATELY TO FAR-ADVANCED CHRONIC OBSTRUCTIVE EMPHYSEMA.

lung membrane (DM) due to breath-holding at full inspiration was considered possible, particularly since the steady state D_L nearly always fell at functional residual capacity (FRC) on the curve relating single breath D_L and V_A. However, experimental increase of FRC in a body plethysmograph failed to increase the steady state D_L.

3. *Variations of the pulmonary capillary blood volume* (V_c) were thought to be largely responsible for both the larger value of the single breath test and for variations with V_A. Experiments with "instantaneous D_L" determinations suggested that the single breath D_L value is a complex of a rapidly rising D_L during the Müller-like maneuver of forced deep inspiration, and a decreasing D_L during the subsequent Valsalva-like breath-holding. Measured peak negative intrathoracic pressure during forced inspiration appeared related to V_c and hence to D_L. Maneuvers tending to increase V_c (Müller maneuver, "G-suit" inflation, recumbency, brief breath-holding, hyperventilation) tend to increase D_L while maneuvers tending to decrease V_c (Valsalva, positive pressure breathing, long breath-holding) decrease D_L.

4. *Inequalities of ventilation, of perfusion or of ventilation:perfusion ratios* were called upon to explain the increasingly greater discrepancy between steady state and single breath D_L in patients with pulmonary disease. In 13 normal subjects the single breath value was, on the average, 1.6 times greater than the physiological dead space steady state D_L; it was 2.3 times greater in 28 patients with a variety of pulmonary fibroses, many of whom had marked lung shunt without complicating emphysema; 2.5 times greater in 38 patients with various sarcoidal reactions some of whom had slight emphysema; and 3.4 times greater in 19 patients with moderate to severe obstructive emphysema. In the last group there was a close relationship between single breath D_L:steady state D_L ratio and the lung nitrogen after 7 minutes' oxygen breathing. It appeared that the two tests did not sample "the same lung."

From the clinical standpoint our experiences suggest that for multiple serial or follow-up studies, the single breath test is useful only when related to the V_A at which the breath was held. Further, an abnormally low single breath D_L implies serious impairment of the apparent diffusing capacity of the lungs while a normal value, particularly in patients with very uneven lungs, does not rule out serious "effective" impairment of the lungs to diffuse O_2 or CO. As a corollary, steady state $D_{L_{CO}}$ or $D_{L_{O_2}}$ values cannot be estimated from single breath $D_{L_{CO}}$.

APPENDIX

In the derivation of the instantaneous D_L given below, comparison is made between algebraic expressions and the graphic presentation of Figure 4:

Let R = dV_A/dt, the rate of inspiration, a constant;

V_A = alveolar volume at any time, t, of inspiration;

V_{CO_i} = volume of CO which has entered the lungs;

Fa_i = fraction of CO which would have obtained from inspired CO alone if diffusion out of the lungs had not occurred;

V_{CO_p} = volume of CO actually present at time t;

V_{CO_D} = volume of CO which has diffused out of lungs;

F_A = actual observed fraction of CO (curve B);

F_I = fraction of CO in inspired gas. If dead space and CO back pressure are ignored then

$$V_{CO_i} = F_I R_t \qquad [1]$$

so that by definition

$$Fa_i = \frac{V_{CO_i}}{V_A}; \quad \text{and} \quad Fa_i = \frac{F_I R_t}{V_A} \quad \text{(curves A)} \qquad [2]$$

Also

$$V_{CO_p} = V_A F_A \qquad [3]$$

Then the volume diffused out is the difference between that which has entered and that which is present:

$$V_{CO_i} - V_{CO_p} = V_{CO_D} \qquad [4]$$

Substituting Equations 1 and 3 into Equation 4:

$$V_{CO_D} = F_I R_t - V_A F_A \quad \text{(curve C)} \qquad [5]$$

Differentiating Equation 5 with respect to t:

$$\frac{dV_{CO_D}}{dt} = F_I R - F_A\left[\frac{dV_A}{dt}\right] - V_A\left[\frac{dF_A}{dt}\right]$$

(slope of curve C) [6]

and since $dV_A/dt = R$

$$\frac{dV_{CO_D}}{dt} = R(F_I - F_A) - V_A\left[\frac{dFa_i}{dt}\right] \qquad [7]$$

But by definition our instantaneous D_L is:

$$D_L = \left[\frac{dV_{CO_D}}{dt}\right] \times \frac{1}{713 \times F_A} \qquad [8]$$

$$D_{L_{inst}} = \frac{R(F_I - F_A) - V_A\left[\frac{dF_A}{dt}\right]}{713 \times F_A}$$

(equation for curve D) [9]

Note that in Equation 9, values for R, F_I, F_A and V_A are known for any given instant from experimental data; dF_A/dt is not, however, and a graphic method for calculation of D_L must still be relied upon.

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ELASTIC PROPERTIES AND THE GEOMETRY OF THE LUNGS *

By JOHN A. PIERCE,† JOE B. HOCOTT AND BILL F. HEFLEY

(From the Department of Medicine, University of Arkansas Medical Center, Little Rock, Ark.)

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The elastic properties of the lung are of primary importance to normal function. A definitive study of these properties entails the simultaneous measurement of pressure and volume under static conditions. A plot of the relationship between pressure and volume in the lungs results in a curve which is approximately linear in the middle part of the vital capacity. The retractive force in this limited part of the curve is a direct function of volume. Although elastic performance is a complex phenomenon, the balance of lung elastic behavior occurs without a delay in time and the majority of the elastic work done on the lung during inspiration can be recovered on expiration. Major contributions are made to the over-all elastic behavior of the lungs by surface phenomena and tissue factors. Factors of minor importance include the bronchial and bronchiolar smooth muscle and the amount of blood in the pulmonary vascular bed.

For many years the elastic properties of the lungs were attributed to elastic tissue fibers. Surface phenomena were not considered important until 1929 when von Neergaard (1) pointed out that the forces arising from the air liquid interfaces throughout the lung parenchyma could be eliminated by the complete replacement of the gas in the lungs with liquid. He demonstrated that pressure was less in the liquid-filled lung than in the air-filled lung at comparable volumes. He assumed an identical operation of tissue elastic factors in air- and liquid-filled lungs.

Radford (2) estimated the surface area of excised lungs, using the principles established by von Neergaard. The difference between air and saline pressure-volume curves during emptying was taken as a measure of the recoverable surface energy. The surface area of the lung was calculated from the recoverable energy. Surface tension was assumed to be 50 dynes per cm. Brown (3) repeated this work. He assumed a value for surface tension at maximal lung inflation. He then calculated a relative value for surface tension at successively lower levels of lung inflation by estimating the change in area associated with the change in volume. In his calculations Brown assumed that the spatial coordinates of all lung units decreased proportionately during deflation.

The purpose of the experiments to be presented was to elucidate further the factors involved in the elastic properties of the lung. The filling of the lungs with mercury metal was studied. It was possible to define the change in surface area of the lungs with accuracy because of the high surface tension of mercury. The equation utilized by Radford (2) was applied in this study:

$$\int_{V_0}^{V_1} P\,dV = \int_{A_0}^{A_1} \gamma\,dA \qquad [1]$$

where P is pressure, V is volume, γ is surface tension and A is area. The right-hand side of the equation was derived from the Helmholtz equation for the free energy of the surface. It was assumed that temperature was constant and that the quantity and composition of the associated bulk phases were independent of area.

Another approach to the problem was to eliminate the tissue elastic properties of the lungs. It was found that after sodium hydroxide extraction and treatment with elastase, the lung no longer exhibited elastic behavior when filled with saline. The pressure-volume measurements with air permitted a study of the geometry of these lung preparations.

MATERIAL AND METHODS

The experiments with mercury filling were carried out as follows. Adult mongrel dogs were given pentobarbital anesthesia (25 to 30 mg per kg of body weight). A Neophor valve was connected to a cuffed endotracheal

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† Investigator of the Arkansas Heart Association.

tube and the animal was ventilated with oxygen at an adjustable positive pressure; expiration was passive at ambient pressure. After opening the chest carefully to avoid injury to the lungs, the trachea was clamped. Virtually all of the gas was absorbed from the lungs prior to cardiac standstill (4). The gas-free lungs were excised and the pulmonary artery and left atrium ligated. A glass connector was secured in the trachea. The preparations were weighed and transferred to a bowl containing mercury metal and isotonic saline solution. Pressure was measured from the tracheal cannula with an open mercury manometer. The lungs were filled with mercury from a glass syringe through an arrangement of three-way stopcocks. The introduction and withdrawal of mercury were performed slowly allowing 3 minutes for equilibration at each volume increment. The pressure in the manometer was related to the surface of the mercury bath. Subpleural mercury spherules were measured with a stereomicroscope at various volumes of lung distention. Care was taken to avoid contact of the mercury-filled lung with the bottom of the bowl, since this elevated the manometer pressure. The lungs were filled to a volume of 500 to 735 ml. The filling and emptying was repeated three times in some of the preparations. At the end of each cycle the lungs were removed and weighed to determine the amount of trapped mercury. The pressure-volume relationships of some of the lungs were studied with saline filling. Preparations were also dried with air and examined microscopically.

A portion of the mercury-filled lung floated above the surface of the mercury reservoir. To correct the pressures in this part of the lung, the volume of exposed preparation was calculated by Archimedes' principle using densities of 1.005 for isotonic sodium chloride solution, 1.05 for lung tissue, and 13.6 for mercury. The area of contact between the lung and the mercury reservoir surface was estimated for various levels of lung distention. The mean transpulmonary pressure in the exposed part of the lungs was equivalent to the pressure at a level equal to one-half the exposed volume divided by the contact area. This correction was small at volumes of distention in excess of 300 ml in the dog lungs. The transpulmonary pressure in the submerged portion of the lung was equal to the difference between the mercury reservoir level and the height of mercury in the manometer. The corrected transpulmonary pressure at any given volume of distention can be expressed as: $(P_m V_i + P_e V_e)/100$ where P_m is the height of the mercury column in the manometer relative to the surface of the mercury reservoir, P_e is the mean transpulmonary pressure in the exposed part of the mercury-filled lung preparation, V_i is the volume percentage of the mercury-filled lung which is immersed, and V_e the volume percentage exposed. The size of the manometer rendered capillarity unimportant.

The interfacial tension between mercury and extracts of tracheobronchial mucus was measured with the drop weight method described by Bartell, Case and Brown (5). The mean results with aqueous and saline solution extracts against mercury were 9 per cent higher than those between mercury and distilled water. Because the intermolecular cohesive forces in mercury are high, the lung-lining layer must penetrate this phase only very slightly, if at all. Other data on the interfacial force area relationships of biological materials against mercury have not been found. As a conservative estimate the calculations in this report were made with an assumed lung mercury interfacial tension of 370 dynes per cm.

The experiments on extracted lungs were performed on dogs of similar body weight. Anesthesia and ventilation were carried out as described before. The lungs were exposed through an extensive transverse incision of the chest. Compliance was determined with a method described previously (6). The lungs were rendered free of gas and then excised. Blood was removed from the pulmonary vessels by perfusion with isotonic saline solution. The vessels were ligated. A glass cannula was tied into the trachea and the lungs were transferred to a vat containing 0.1 N sodium hydroxide solution. This solution was poured into the trachea under a pressure which never exceeded 10 cm of water. Fresh sodium hydroxide solution was replaced frequently over a period of 3 to 5 days with repeated filling of the lungs.

The lung preparations were rinsed with water followed by carbonate buffer at pH 8.8 (7). Crystalline elastase was dissolved in carbonate buffer (16 mg per L). The lung was filled with and immersed in this buffered enzyme solution. The preparation was incubated at 37° C for 18 to 24 hours at the end of which time the pH of the solution varied between 8.6 and 9.1. The lung preparations were drained of fluid and inflated with air. After determining that the preparations would hold air, pressure-volume relationships were measured with a stepwise inflation and deflation. In order to assess the flow-resistive characteristics of these preparations, the time course of passive collapse was also studied. Flow was recorded from a conventional pneumotachograph and pressure was measured with a strain gauge transducer. Surprisingly little air remained trapped within the lung preparations following passive deflation. This trapped gas was not measured but has been estimated as less than 30 ml.

The preparations were transferred to a vat containing a mixture of ethanol and ether (3:1 by volume). This solution was poured into the trachea. After 30 to 45 minutes in this bath they were drained of liquid and pressure-volume relationships were again measured with air inflation. Studies of passive collapse were repeated. Gas volumes reported are at an ambient temperature of 24 ± 2° C.

The lung preparations were subsequently immersed in normal saline solution at 37° C. The pressure-volume relationships were determined during a stepwise distention with saline solution. Because some solution escaped during the procedure, it was necessary to estimate the final volume from the weight of the preparations. The intermediate volumes were calculated as though saline had escaped at a regular rate. It was demonstrated that saline escape occurred predominantly during filling, since the volume of distended preparations did not change appreciably over periods up to an hour. Thus,

TABLE I

Studies on mercury-filled dog lungs

Animal no.	Body wt	Wt of lungs*	Maximal volume†	Pressure at maximal volume	Pressure at 400 ml volume	On emptying from 400 ml volume: Work recovered‡	On emptying from 400 ml volume: Surface area	Pressure at 200 ml volume	Compliance	Volume of trapped mercury
	kg	*g*	*ml*	*mm Hg*	*mm Hg*	*10^6 ergs*	*m^2*	*mm Hg*	*ml/cm H_2O*	*ml*
1	8.9	108	500	106	82	24.2	6.54	51	6.1	27
2	10.2	167	500	96	78	24.2	6.54	47	5.1	15
3	6.4	107	600§	112	69					
			550	94	68	17.7	4.78	37	4.7	42
			592	103	68	18.6	5.03	38	5.7	61
4	7.3	104	735	84	45	10.7	2.89	34	6.8	82
5	5.7	109	500	95	74	22.0	5.95	45	5.5	21
			521	96	68	19.0	5.14	39	4.7	32
			532	98	67	16.9	4.57	36	5.1	49
6	10.9	137	600	93	68	21.2	5.73	44	6.8	29
			621	89	61	16.4	4.43	36	7.0	23
			625	90	62	18.2	4.92	37	6.3	43
Mean	8.2	122	573	96	67	19.0	5.14	40	5.8	38.5
SD‖	2.1	25			9	3.9	1.05	5	0.9	

* Includes both lungs and a segment of trachea. † Volumes refer to mercury contained in the lungs. ‡ As measured with a planimeter (see Figure 1). § The lungs were only partially emptied on this cycle. ‖ Standard deviation as $\sqrt{\frac{\Sigma(D^2)}{N-1}}$.

the pressures reported are reliable static measurements. Further, the contained saline could not be aspirated from the trachea so that data were obtained only during filling.

Finally, small sections of the preparations were fixed in formalin for histological examination with Verhoeff's elastic tissue stain. The balance of each preparation was stored at $-4°$ C and subsequently analyzed for collagen and elastin with a modification of the procedure described by Lowry, Gilligan and Katersky (8). The modified method has been described previously (9) with the single exception that the initial overnight sodium hydroxide extraction was omitted, since the native lungs had been exposed to alkali.

RESULTS

Table I presents results of the mercury studies in detail. The mean transpulmonary pressure at maximal distention was 96 mm of mercury. Work recovered on emptying the mercury-filled lungs was calculated from a 400 ml contained volume (Figure 1). This volume was chosen arbitrarily to approximate the functional residual capacity, and the surface area of the lungs was calculated from this level of distention. The mean compliance of the mercury-emptying curves was approximately one-eighth that found in air-filled lungs. This indicates an eightfold increase in retractive force in the mercury-filled lung if the surface areas are assumed comparable with mercury and air filling at similar volumes of distention. Static measurements of pressure and volume with saline filling were similar before and after mercury filling. Gross and microscopic examination of air-dried specimens revealed no severe distortion of lung architecture after mercury filling.

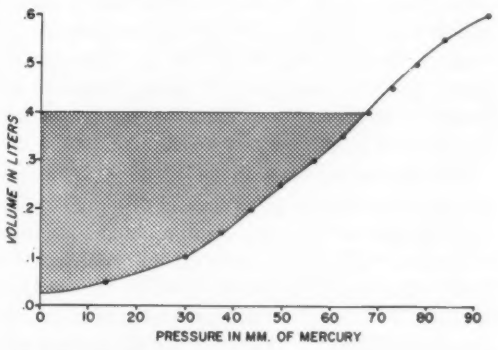

FIG. 1. STATIC PRESSURE-VOLUME CURVE OBTAINED ON EMPTYING THE MERCURY-FILLED LUNGS. The shaded area was measured with a planimeter to estimate recovered surface work. This recovered work was used in Equation 1 to calculate lung surface area.

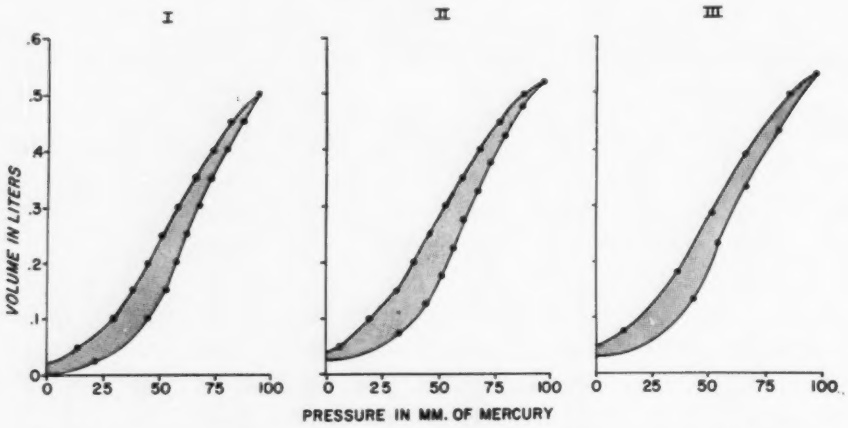

FIG. 2. SUCCESSIVE STATIC PRESSURE-VOLUME CURVES OBTAINED FROM A SINGLE PAIR OF LUNGS DEMONSTRATING THE REPRODUCIBILITY OF THE MEASUREMENTS. The shaded areas depict the differences observed between filling (lower curved line) and emptying (upper curved line) and referred to as hysteresis.

Emphasis has been placed on the measurements during emptying of the lung because only during this part of the cycle is it possible to recover work and hence, estimate lung surface area. Figure 2 represents data obtained on filling and emptying the lungs with mercury in Dog 5. The results shown are typical of those obtained in all animals. Hysteresis was regularly observed. It has been attributed to the irregular opening of terminal air spaces in various parts of the lung. This causes a discrepancy in the distribution of the contained volume on filling as opposed to that on emptying. Relative to the work recovered on emptying the lung, hysteresis was roughly similar in the mercury studies and in the elastase-treated lungs. Whether this hysteresis is related to the purely physical nature of the lining surface of the lungs or whether some chemical reaction might be involved is not entirely clear.

Measurements were made of the diameter of subpleural mercury spherules as observed in the exposed part of the mercury-filled lung. Physical limitations of the stereomicroscope required that these spherules be selected from a flat exposed surface. The results of these measurements are shown in Table II. It should not be inferred that these spherules were necessarily representative of all terminal air spaces. The largest spherules occurred at low volumes of distention while the smallest subpleural spherules were seen only with greater filling. It should be noted that these observations were made at levels of distention near the functional residual capacity and hence would not compare with fully expanded lung preparations.

Table III presents data on the experiments with alkaline extraction and elastase treatment of the lungs. Tissue elastic behavior has been assessed customarily in lungs filled with saline solution. Figure 3 presents such an assessment on a single pair of lungs from a dog. These results were typical of those obtained in several similar experiments. Curve I in Figure 3 presents static pressure-volume data obtained on emptying the lungs which were freshly excised, free of gas and filled with isotonic saline solution. Curve I thus represents the normal tissue elastic behavior in excised dog lungs. Curve II in Figure 3 was obtained in an identical manner after immersion for

TABLE II

Measurements of subpleural mercury spherules

Volume	No. of observations	Mean diameter	Range
ml		μ	μ
100	19	266	120–400
200	31	185	120–320
300	18	142	80–220
400	31	121	60–240
500	9	96	80–160

TABLE III

Studies on dog lungs extracted with alkali and exposed to elastase

Animal no.	Body wt	Compliance *in vivo* chest open	Measured with air on deflation following alkali and elastase: Rinsed with water	Rinsed with ethanol-ether	Pressure at 400 ml volume	On deflation from 400 ml volume: Work recovered	Surface area	Compliance under saline*	Elastin†
	kg	*ml/cm H_2O*	*ml/cm H_2O*	*ml/cm H_2O*	*cm H_2O*	*10^6 ergs*	*m^2*	*ml/cm H_2O*	%
7	9.5	40	25	62					
8	9.5	42	32	73	16.6	3.22	6.44	>1,000	1.9
9	10.2	52	38	123	12.5	2.69	5.38		
10	12.3	64	45	155	11.9	1.67	3.34		
11	8.0	37	40	71	11.6	2.47	4.94	>1,000	5.7
12	8.2	41	26	53				>1,500	
13	6.5	35	26	37	18.5	3.13	6.26	>1,500	4.1
14	10.5	61	55	118	11.0	2.58	5.16	>1,500	
Mean	9.3	46.5	36	86.5	13.7	2.63	5.25		3.7
SD‡	1.8	11	11	41	3.1	0.56	1.11		

* With saline solution following extraction with alkali and exposure to elastase; measurement completed on only 5 animals. † Expressed as percentage of normal assuming a ratio of collagen to elastin of 3; the analyses were done in groups. ‡ Standard deviation as $\sqrt{\frac{\Sigma(D^2)}{N-1}}$.

3 days at room temperature in 0.1 N sodium hydroxide solution. The same lungs were used in all curves of Figure 3. Any given volume of distention on curve II results in approximately one-half the pressure observed in freshly excised lungs (curve I). The lungs were treated with elastase in buffer for 24 hours and pressure-volume data were again obtained under saline immersion (curve III, Figure 3). These measurements were static but were made on filling, since saline could not be aspirated from the tracheal cannula. The resultant pressure in curve III is less than 10 per cent of that observed at comparable volumes of distention in the freshly excised lungs. The increasing slope of the pressure-volume diagram from curve I to II to III represents an increasing compliance of the saline-filled lung. More prolonged extraction in alkali or extraction in more concentrated (0.2 N) sodium hydroxide solution resulted in additional increases in saline compliance. Thus, the loss of elastic behavior by the lung tissue has not been considered a specific result of elastin hydrolysis. The changes observed with exposure to alkali are interpreted as evidence of a progressive loss of tissue organization.

The maximal increase in saline compliance occurred after treatment with elastase in buffer. For this reason the elastase-treated lungs have been considered as devoid of tissue elastic behavior. When such preparations were filled with air, the pressure-volume behavior was similar to that observed in native air-filled lungs. Figure 4 presents a typical static pressure-volume diagram on air inflation and deflation of an alkali- and elastase-treated dog lung preparation. The pressure-volume relationships in Figure 4 do not differ markedly from those measured *in vivo* with the

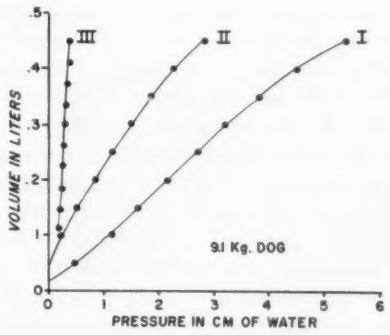

FIG. 3. STATIC PRESSURE-VOLUME CURVES WITH SALINE SOLUTION OBTAINED FROM A SINGLE PAIR OF DOG LUNGS. The curves are: I, before treatment; II, after exposure to sodium hydroxide; and III, after treatment with elastase. Curves I and II were obtained on emptying and curve III was obtained on filling the lungs with saline solution.

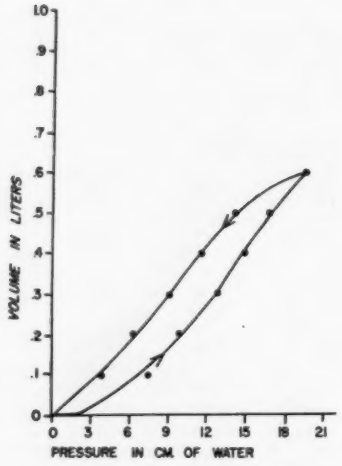

FIG. 4. TYPICAL STATIC PRESSURE-VOLUME DIAGRAM OBTAINED ON AIR INFLATION AND DEFLATION. The lungs had been exposed to sodium hydroxide and elastase solutions. A small volume of trapped air has been ignored (see text).

chest open. Air compliance *in vivo* was not significantly different from that found after alkali and elastase treatment (Table III). There was a difference in the amount of gas trapped on emptying, being much greater *in vivo* than in the alkali- and elastase-treated lungs. The striking feature is that the preparation shown in Figure 4 exhibited virtually no tissue elastic behavior when studied with saline filling. Figure 5 presents a pressure-volume curve obtained from static measurements of another air-filled dog lung preparation following treatment with alkali and elastase. Figure 5 also includes a pressure-volume diagram of an elastase-treated preparation after exposure to ethanol ether solution. It was estimated that this treatment should reduce the lung surface tension by one-half. The surface work recovered on deflation from a 400 ml volume was compared in the water-rinsed and ethanol ether-treated preparations. Ethanol ether treatment reduced the recovered surface work by 30 per cent in six preparations.

Histologic examination of the elastase-treated preparations revealed only scanty amounts of elastic tissue as compared with preparations treated similarly but without elastase. Moreover, chemical analyses revealed only 4 per cent of the expected normal amount of elastin. There is no doubt that elastic tissue fibers, as they usually exist in the lungs, were almost completely absent from these preparations. Compliance under saline was not found to change following 18 to 24 hours' incubation in carbonate buffer at pH 8.8 in the absence of elastase.

Passive collapse records with air, before and after elastase treatment, showed no appreciable differences. This indicates that the flow-resistive characteristics of the lung preparations were not greatly altered by treatment with elastase and implies that the geometry of the terminal air spaces was not severely disordered. Examination of elastase-treated preparations dried with air revealed architecture similar to that found in untreated lungs.

DISCUSSION

The studies with mercury metal are suitable for evaluation of the geometry of the terminal air spaces of the lung. This liquid has an extremely high surface tension and is essentially immiscible with the lining layer of the lungs. Thus the mercury-filled lung has an unusually large interfacial tension and produces a large retractive force on distention. The pressure on distention is sufficiently great that the small pressure observed in the saline-filled lung and attributed to tissue elastic performance becomes negligible. Interfacial tension has not been measured within the mercury-filled lung, but probably would approximate the interfacial tension of mercury measured against extracts of tracheobronchial mucus. Although the question is unsettled whether interfacial tension might vary with changes in interfacial area, a tension decrease of 40 dynes per cm would alter the present results by only 10 per cent. Thus the static pressure-volume characteristics of the mercury-filled lung appear to result from surface phenomena.

If it can be assumed that the pressure-volume relationships of the mercury-filled lung are a reflection of surface phenomena at the mercury lung interface, then the surface area of the lung can be readily calculated. The estimation of surface area is based on the familiar equation relating work to surface area (Equation 1). The pressure-volume curve on emptying is used in the calculation as the distention curves are modified by irregular filling in different portions of the lung. The method of estimating recoverable surface work is

TABLE IV

*Physical measurements of the surface area of the lungs of dogs**

Author	Year	Method	Surface area
			m^2
Radford (2)	1954	Air and saline emptying	0.5
Brown (3)	1957	Air and saline emptying	10.3
Present study		Mercury metal emptying	5.1
Present study		Air emptying of alkali- and elastase-treated lungs	5.2

* At 400 ml volume.

illustrated in Figure 1. The interfacial tension at the lung mercury junction was taken as constant at 370 dynes per cm. The mean values for surface area obtained in the dog lungs are compared with the results of other investigators in Table IV.

The alkali-extracted elastase-treated lung offers a similar opportunity to define the geometry of the lung. This lung preparation exhibits essentially no elastic properties when filled with saline. From this, it is reasonable to conclude that the pressure-volume characteristics with air are a reflection of the changing air fluid interfaces with inflation and deflation. If we assume a surface tension of 50 dynes per cm for the lungs washed with water, we can calculate the surface area and the ratio of area to volume at different levels of inflation, using Equation 1. While the value chosen for surface tension is not as secure as in the case with mercury, it appears reasonable. It is also necessary to assume that the surface tension does not vary with inflation and deflation of the lungs. The material described by Clements, Brown, and Johnson (10) may have been removed by sodium hydroxide extraction and repeated water rinsing. The mean value for surface area obtained from the elastase-treated lungs at a 400 ml inflation (5.2 m^2) agrees remarkably well with the data from the mercury-filled lung (5.1 m^2).

In both types of study the effects of surface phenomena have been exaggerated. This is true in the mercury-filled lung because of high interfacial tension and in the elastase-treated lung because of impaired tissue elastic performance. Neither preparation is exactly equivalent to the lungs during life. Abnormally large pressures existed across the mercury-filled lungs which must have imposed a strain on supporting structures such as the connective tissue fibers. The pressure exerted on the wall of any given air space depends on the major radii of curvature of the space and on the existing pressure within the lungs. With the smallest radius of curvature, one might expect negligible pressure on the wall. This occurs where the radius of curvature and the interfacial tension precisely satisfy the Laplace theorem. That is, $P = 2\gamma/r$, where P is pressure, γ is surface or interfacial tension and r is radius. Where the radius is greater there will be pressure transmitted to the wall and hence tension on the supporting framework of the lungs. The actual stress on the framework of the lung might lead to distortion or destruction of the normal lung structure. The elastase studies are open to the same criticism because the connective tissue has been altered. Several observations suggest that this error does not invalidate the present results. It was possible to fill and empty the lungs repeatedly without significantly altering the data obtained. This was

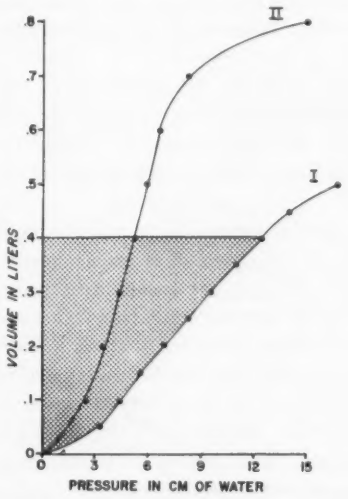

FIG. 5. STATIC PRESSURE-VOLUME CURVES WITH AIR OBTAINED ON EMPTYING AN ALKALI- AND ELASTASE-TREATED PREPARATION. The shaded area depicts the recovered surface work from 400 ml for curve I, after water rinse. Curve II was obtained following ethanol ether rinse to reduce the surface tension in the lungs. A small volume of trapped air has been ignored (see text).

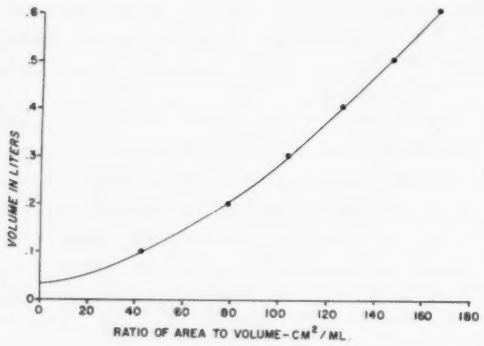

FIG. 6. A PLOT OF THE RELATIONSHIP BETWEEN VOLUME AND THE RATIO OF LUNG SURFACE AREA TO VOLUME, AS MEASURED WITH MERCURY METAL.

true in both types of study. Examination of specimens inflated and dried with air failed to reveal evidence of marked dilatation of the proximal air spaces. These examinations were performed following mercury filling and in preparations treated with elastase. It seems unlikely that the larger air spaces could withstand extreme overexpansion without rupture of the connective tissue fibers in their walls. Widespread rupture of these fibers should have been associated with changes in pressure-volume relationships and alterations in the appearance of air-dried inflated specimens. Finally, measurements of the size of subpleural mercury spherules are in general agreement with the measurements of Radford and McLaughlin (11) made on dog lungs during air inflation.

The elastase-treated lungs afford a striking example of the effects of surface phenomena. These lungs had virtually no tissue elastic behavior and yet retained normal relationships between pressure and volume on filling with air. Under these circumstances surface factors must have accounted for virtually all of the observed elastic behavior. This contrasts sharply with the findings in normal lungs. Tissue elastic performance measured by saline-emptying curves in excised lungs amounts to about one-half of total elastic behavior during inflation with air. The exact role of tissue elasticity, however, is far from clear. Interfacial tensions undoubtedly exist at cell boundaries and elsewhere in the alveolar wall. The importance of such interfaces has been discussed by Thompson (12) although they have not been evaluated in the lungs. Pressure-volume measurements of the lungs do not distinguish between the elastic behavior arising in tissue fibers and that resulting from such interfacial tensions. Although elastase treatment abolished tissue elastic performance as measured with saline distention, this in no way proves that the elastic fibers are solely responsible for these properties since the enzyme also probably destroys tissue boundaries and eliminates the effects of interfacial tensions. Thus, the precise role of the elastic fibers is not completely defined.

The size of the air sacs also has importance. Any regularly shaped geometric figure has a unique ratio of surface to volume at every different size. An increase in the radius of curvature leads to a decrease in this ratio. In considering the entire lungs, the ratio of surface to volume must reflect the mean size of the spaces in the lungs. Figure 6 presents the ratio of surface to volume calculated on emptying the mercury-filled dog lungs. It is of interest that this ratio decreases as the volume diminishes. The elastase-treated lungs gave similar results. This can only be interpreted to indicate that the mean radius of curvature of the air spaces increased as volume was removed from the lungs. This must have resulted from the closure of some of the smaller air spaces. In the mercury-filled lungs, this was documented by the measurements of subpleural mercury spherules at various levels of distention. Radford and McLaughlin (11, 13) obtained results similar to these on air inflation and deflation after surface-active solutions had been introduced into the terminal air spaces of the lungs of rats. Their observations on the alveolar behavior of freshly excised lungs, however, indicated rather marked discrepancies between inflation and deflation.

Several observations suggest that the lungs are lined by a fluid layer with intense surface activity. Macklin (14) has discussed this in detail, citing the report of Terry (15), who in 1926 observed a clear free fluid in the alveoli during life. Macklin visualized this material as facilitating the removal of inhaled particles and suggested that the fluid was produced by the granular pneumonocytes. Pattle (16) noted an unusual stability in the foam of pulmonary edema fluid and in fluid expressed from the cut surface of fresh lung tissue. He con-

cluded that the stability of the foam was due to an insoluble surface layer of protein that arose from the lungs. Clements (17) described the surface characteristics of extracts of lungs and tracheobronchial mucus. This material exhibited marked hysteresis on expansion and compression when studied as an adsorbed film in a surface balance. Similar effects were not observed with extracts of defibrinated blood plasma. Chase (18) studied lung tissue which had been rapidly frozen and demonstrated a homogeneous film on the surface of the alveoli in electron photomicrographs. This material was positive with the periodic acid-Schiff stain. The chemical nature of this material has not been clarified, although it is thought to be a polysaccharide protein complex. Clements, Brown, and Johnson (10) have pointed out that the material observed in the film balance could extend the range of stable volume behavior for individual alveoli and permit the coexistence of alveolar spaces of widely varying sizes in the lungs. They describe this material as having "antiatelectasis" properties. There seems little room for doubt about the existence of an alveolar lining layer which has intense surface activity.

Such are the factors which govern the elastic behavior of the lungs. Information about these factors gained in diverse ways can not be transferred directly to the conditions which exist during life. Thus, the central problem now concerns the relative importance of the various factors already discussed. In our opinion, the size of the air cells is critical in providing a properly curved surface to produce the retractive force necessary to expel air from the lungs. The material lining the lungs may delay the closure of small air spaces with minor changes in pressure and thus lessen the work required to fill the lungs on tidal breathing. The connective tissue framework of the lungs may not participate at all in lung elastic behavior at small volumes of distention. At large volumes, however, these fibers supply the strength necessary to retain the structure of the lungs and thus avoid permanent deformity. Additional studies will be required to determine whether these views are correct.

SUMMARY

1. The elastic properties of dog lungs were studied with mercury filling and after treatment with elastase. The effects of surface phenomena were dominant in both of these experiments.

2. Estimates of lung surface area from measurements of recovered surface work were in agreement by these two methods.

3. In the mercury studies, the over-all ratio of surface to volume decreased and the size of subpleural mercury spherules increased as the lung was emptied; the reverse occurred on filling.

4. The studies with elastase-treated lungs served to show that elastic fibers are not essential for normal elastic performance on inflation with air.

5. Those factors important to the normal pressure-volume characteristics of the lungs were considered in some detail. We believe the size of the terminal air spaces is the single most important consideration.

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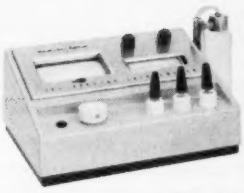

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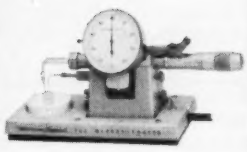

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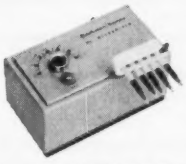

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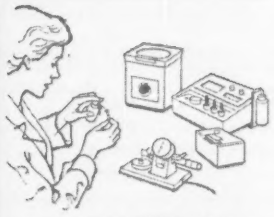

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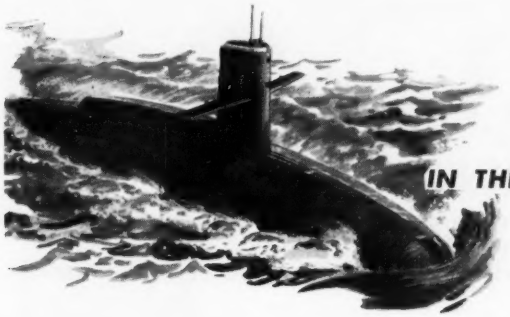

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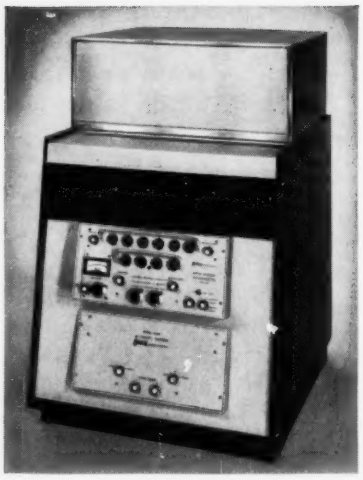

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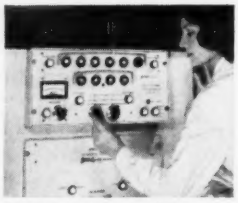

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PART 2

VOLUME 40
NUMBER 8

AUGUST, 1961

TWO PARTS
PART II

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POST-STREPTOCOCCAL GLOMERULONEPHRITIS: HISTOPATHOLOGIC AND CLINICAL STUDIES OF THE ACUTE, SUBSIDING ACUTE AND EARLY CHRONIC LATENT PHASES * †

By ROBERT B. JENNINGS and DAVID P. EARLE

(From The Departments of Pathology and Medicine, Northwestern University Medical School and the VA Research Hospital, Chicago; and the Departments of Medicine of the US Naval Hospital, Great Lakes, and Passavant Memorial Hospital, Chicago, Ill.)

(Submitted for publication November 30, 1960; accepted January 23, 1961)

Most present knowledge of structural changes in the kidneys of patients with clinical acute glomerulonephritis has come from postmortem studies made on the occasional patient who died during the acute phase of the disease. Such studies, particularly those of Langhans (1), Löhlein (2), Volhard and Fahr (3), Bell (4), McGregor (5) and others (6, 7), have given an accurate account of the pathology of severe acute glomerulonephritis and have formed the present source of textbook description.

Mortality during acute glomerulonephritis has been variously estimated as between 2 and 12 per cent (8–13). Another 1 or 2 per cent of patients has died within a year or so after onset, having followed a subacute or persistently active clinical course (2, 3, 9). Most authors have assumed that the renal changes found in the majority of patients who survived the initial acute attack would be qualitatively similar to those noted in the fatal cases, although little direct evidence existed to support this view. Furthermore, it is not known whether any distinctive histologic features characterize the kidneys of those patients who completely recover clinically as opposed to those who develop chronic latent glomerulonephritis.

These problems can now be studied by correlation of the clinical course of glomerulonephritis with structural changes found on percutaneous renal biopsy. A few cases studied by this technique have been published (14–21) and some of our own results have been reported previously in preliminary form (22–24).

The present paper is a report of clinical and histological observations in 36 patients with sporadic clinical acute glomerulonephritis proved in each instance to have followed a Group A hemolytic streptococcal infection. One purpose is to describe in detail the types of lesions found in the kidneys during the acute phase of this disease and to illustrate how they change with time. A second and perhaps more important purpose is to present preliminary data on the types of lesions most commonly associated with the development of early clinical chronic glomerulonephritis. The results suggest that acute post-streptococcal glomerulonephritis is a readily definable disease with fairly characteristic histologic abnormalities. Post-streptococcal clinical glomerulonephritis, both during the acute and early chronic phases, usually can be distinguished from glomerular diseases of non-streptococcal etiology.

MATERIALS AND METHODS

The criteria for selection of patients for a study of the natural history and pathology of acute glomerulonephritis must be rather rigid, since a variety of different primary and secondary glomerular diseases may masquerade under this clinical diagnosis (22, 23, 25).

Patients. The present report is based on observations on 36 patients who presented clinical and laboratory evidence of acute glomerulonephritis or both. The disease followed an infection in 32 patients which was proved to be of hemolytic streptococcal etiology by isolation of Group A hemolytic streptococci on culture and/or by a significant increase in serum antistreptolysin O (ASO), antistreptokinase (ASK) or antihyaluronidase (AH) titers on serial measurement (Table I). History of infection was not obtained in 4 patients but serial changes in ASO, ASK or AH titers indicated a preceding strep-

* This work was supported in part by Grants H-1815 and H-1890 from the National Heart Institute and from the Otho S. A. Sprague Foundation.

† Publication of this supplement was made possible by a grant-in-aid from the Merck Sharp & Dohme Postgraduate Program.

TABLE I

Summary of certain clinical data in patients with acute glomerulonephritis [a]

Patient	Age	Sex	Prior known normal urine	Associated infection	Throat culture	Serum ASO (units) Max.	Serum ASO (units) Min.	Serum T-12 antibodies	Latent period (days)	Comments
						Healed				
Swi	22	♂	11 D	Tonsillitis	BHS	500	100	pos.	25	Hyperthyroidism. Urine normal 14 D after infection
Dil	18	♂	1 M	Cellulitis	ND	2,000	625	neg.	3	Transient erythema multiforme
Ros	20	♂	±1 Y	Pharyngitis	neg.	500	125	neg.	7	Only test for T-12 antibodies at 6 W
Sch	18	♂	3 W	Pneumonitis	BHS	333	125	neg.	±14	Right lower lobe pneumonitis at onset
Shi	20	♂	1 M	Pharyngitis	BHS	166	(4 M)	neg.	±12	Petechiae at onset
Gav	20	♂	±1 Y	Tonsillitis	ND	250	100	pos.	21	First ASO titer at 3 M
Swe	18	♂	±6 M	Pharyngitis	ND	166	50	neg.	6	
Eas	22	♂	3 M	Pharyngitis	ND	333	100	neg.	3	Pneumonitis on roentgenogram at onset
Ser	40	♂	2 Y	Pharyngitis	neg.	500	166	neg.	12	
Mol	18	♂	12 D	Tonsillitis	ND	625	50	neg.	20	Urine normal 8 D after infection
Dix	22	♂	1 M	Uncertain	BHS	333	ND	ND		Pilonidal cyst drained 1 M before; no resp. infect.
Mul	43	♀	None	Pharyngitis	NHS	100	50	pos.	±23	Six others of family developed pharyngitis (B hemol. strep.); these could not be typed but patient and 2 others developed serum antibodies against T-12; 3 of patient's children also developed mild acute glomerulonephritis
Moe	18	♂	±1 Y	Pharyngitis	neg.	333	125	neg.	10	Right lower lobe pneumonia with effusion at onset
Vin	32	♂	4 Y	Tonsillitis	T-12	2,560	625	neg.	17	
Har	30	♀	None	Pharynigtis	ND	333	125	pos.	18	
Roo	66	♂	3 Y	None noted	ND	333	50	neg.		Renal calculus, bladder tumor (successfully removed), mild diabetes
Col	39	♂	None	Pharyngitis	ND	250	83	pos.	14	Chronic dermatitis, type unknown
Eva	18	♂	4 D	Pharyngitis	ND	1,000	200	pos.	11	Three Y, and again 5 D before onset had acute rheumatic fever, no cardiac involvement

[a] Abbreviations: ASO = antistreptolysin titer. ASK = antistreptokinase titer. AH = antihyaluronidase titer. T-12 = Type 12 hemolytic streptococcus. BHS = beta hemolytic streptococcus. ND = Not done. gr.AHS = Group A hemolytic streptococcus. D = days; W = weeks; M = months; Y = years.

TABLE I—(*Continued*)

Patient	Age	Sex	Prior known normal urine	Associated infection	Throat culture	Serum ASO (units) Max.	Serum ASO (units) Min.	Serum T-12 anti-bodies	Latent period (days)	Comments
						Healed				
Eik	48	♂	None	Pharyngitis	neg.	333	125	pos.	21	Also had cellulitis; chronic alcoholism
San	24	♂	None	Pharyngitis	neg.	500	50	neg.	40	*S. mansoni* infestation, duodenal ulcer
McC	59	♂	None	Pharyngitis	neg.	500	125	neg.	±10	Arteriosclerotic heart disease
Lee	44	♂	None	"Flu"	neg.	250	100	neg.	±38	Chronic active pulmonary tuberculosis; atypical photochromogenic organism
Mar	57	♂	2 Y	None noted	neg.	625	250	neg.		
						Not healed at last follow-up				
Dra	20	♂	±2 Y	Pharyngitis	ND	250	50	pos.	28	
Byr	17	♂	±6 M	Pharyngitis	ND	500	50	pos.	6	Urticaria after benzathine penicillin G given for pharyngitis
Lar	27	♂	±2 Y	Pharyngitis	ND	125	50	neg.	21	
Rob	20	♂	±2 Y	None noted	ND	166	125	pos.		AH titers 640, 320; ASK titers 1448, 512
Bis	21	♂	±1 Y	Pharyngitis	BHS	125	100	neg.	33	First ASO at 4 M; AH titer 512
Jam	26	♂	7 M	Tonsillitis	T-12	200	83	pos.	14	
Win	20	♂	±1 Y	Pharyngitis	neg.	333	250	ND	15	Only patient in series with positive urine cultures; *E. coli* once *A. aerogenes* twice
Gar	10	♀	None	Pharyngitis	gr. AHS	333	62	neg.	14	
Lat	19	♂	3 W	Pharyngitis	ND	333	50	neg.	42	Transient purpura and arthralgia at onset
Ape	37	♀	1 Y	Pharyngitis	gr. AHS	833	200	neg.	10	
Vla	37	♂	6 M	Pharyngitis	ND	333	100	neg.	28	Pneumonia 21 M after onset; appendectomy 30 M after onset
Tra	19	♂	3 M	Pharyngitis	ND	500	125	neg.	±28	
						Died				
Cal	34	♂	None	Pharyngitis	neg.	400	333	ND	4	Died 32 D after onset

TABLE II

Data concerned with renal disease in patients with acute glomerulonephritis *

Patient	Presenting renal symptoms	Edema	Maximum blood pressure	Urine: Onset Prot.	Onset RBC	Duration Prot.	Duration RBC	Final Prot.	Final RBC	Maximum BUN	Minimum PSP	Duration follow-up	Comments
			mm Hg							*mg %*	*%*	*months*	
							Healed						
Swi	(Urinalysis)	0	140/60	1+	mod.	9.5 M	9.5 M	0	0	12	35	17	
Dil	(Urinalysis)	0	124/84	2+	mod.	7 W	4 M	0	occ.	14		4	
Ros	Gross hematuria	0	120/70	0	gross		1 M	0	0	15	20	5	All urines protein free
Sch	(Urinalysis)	0	158/100	3+	occ.	5 M	4 M	0	0	15	30	7.5	
Shi	Gross hematuria	0	130/80	1+	gross	3.5 M	3.5 M	0	0	17	58	5	
Gav	Gross hematuria	1+	140/80	3+	gross	2.5 M	1 M	0	0	18	35	4	
Swe	Gross hematuria	0	120/90	2+	gross	2.5 M	2.5 M	0	0	20	30	4	
Eas	Gross hematuria	0	146/100	4+	gross	3 M	3 M	0	0	20	25	19	
Ser	Gross hematuria	3+	172/116	4+	gross	3 M	3.5 M	0	0	22	24	26	Transient hematuria at 24 M during Salmonella dysentery
Mol	(Urinalysis)	0	138/60	4+	many	2.5 M	3.5 M	0	0	24	35	8	
Dix	Gross hematuria	0	160/95	3+	gross	2.5 W	2.5 M	0	0	24	25	3	
Mul	Gross hematuria	2+	160/100	3+	gross	4 M	7 M	0	occ.	24		8.5	
Moe	(Urinalysis)	0	180/120	3+	many	8–24 M	7 M	0	0	25		25	
Vin	Gross hematuria	2+	204/116	4+	gross	1 M	1 M	0	0	25	20	4	
Har	Edema	3+	170/108	4+	many	2 M	2 M	0	0	25		6	Steroid therapy begun 2nd month; urea clearance increased from 10 to 46% of normal in 2 W
Roo	Edema	3+	160/90	3+	many	11 M	6 M	0	0	27	2	36	PSP 12%, urea clearance 61% of normal at 5 M
Col	Gross hematuria	3+	166/118	2+	gross	26 M	4 M	0	0	28	21	51	Steroid therapy for 2 M, beginning 5 M after onset; urea clearance increased abruptly from 47 to 80% of normal during therapy
Eva	(Urinalysis)	0	130/90	2+	many	1 M	6.5 M	0	many	33		6.5	
Eik	Edema	4+	200/110	4+	many	1.5 M	1.5 M	0	0	35	20	5	Urea clearance normal at 1 M
San	Gross hematuria	1+	180/110	4+	gross	4 M	16 M	0	0	42	14	26	PSP 36% at 15 M; urea clearance 103% of normal at 19 M

* Abbreviations: Prot. = protein. RBC = red blood cells. Y = year, M = months, W = weeks, D = days. BUN = blood urea nitrogen. PSP = phenolsulfonphthalein excretion, per cent in 15 minutes.

TABLE II—(*Continued*)

Patient	Presenting renal symptoms	Edema	Maximum blood pressure	Urine: Onset Prot.	Onset RBC	Duration Prot.	Duration RBC	Final Prot.	Final RBC	Maximum BUN	Minimum PSP	Duration follow-up	Comments
			mm Hg							*mg %*	*%*	*months*	
						Healed							
McC	Edema	2+	200/110	3+	many	3 W	4 W	0	0	45		36	Urea clearance 73% of normal at 4 M
Lee	Edema	3+	126/86	3+	gross	3.5 M	5 M	0	0	66		7	
Mar	Edema, oliguria	2+	175/95	2+	many	4 M	4 M	0	0	128		6	Steroid therapy begun 1 M after onset, for 1 M; urea clearance had been minimal, increased to 29% of normal at end of treatment
						Not healed at last follow-up							
Dra	Gross hematuria	2+	135/85	4+	gross	10+ M	10+ M	4+	many	15	16	10	Developed nephrotic syndrome at 7 M
Byr	Costovertebral tenderness	0	124/80	1+	mod.	4.5+ M	4.5+ M	4+	0	17	15	4.5	Proteinuria increased during observ. period despite bed rest; PSP 30% at 3 M
Lar	Gross hematuria	1+	220/120	4+	gross	37+ M	37+ M	1+	occ.	22	15	37	At 17 M 3 of 13 urines had protein
Rob	Gross hematuria	3+	164/120	4+	gross	4+ M	4+ M	1+	many	24		4	Killed in accident 1 Y after onset; no autopsy
Bis	Gross hematuria	2+	168/110	4+	gross	7+ M	7+ M	1+	occ.	24		7	
Jam	(Urinalysis)	0	134/90	3+	many	40+ M	40+ M	2+	occ.	25	6	40	Hypertension (132/90 to 190/118) during follow-up
Win	Edema	4+	180/110	2+	many	4+ M	4+ M	1+	occ.	39		4	BUN still increased at last observation
Gar	Edema	1+	200/120	4+	few	16+ M	16+ M	1+	few	40		16	
Lat	Edema	2+	138/98	4+	many	45+ M	45+ M	1+	rare	45		45	
Ape	Edema	4+	180/130	4+	many	25+ M	25+ M	2+	many	79	1	25	
Vla	Edema	3+	170/100	4+	many	31+ M	31+ M	1+	few	96	0	31	Steroid therapy 2nd M; marked improvement in urinary findings and anemia during therapy; urea clearance increased from 19 to 73% of normal
Tra	Gross hematuria	0	160/98	4+	gross	8+ M	8+ M	1+	few	200		8	Pericardial friction rub; plasma potassium = 7.0 mEq/L; steroid therapy 1st M associated with dramatic improvement in clinical symptoms, biochemical abnormalities and urine
						Died							
Cal	Gross hematuria	4+	180/116	4+	gross	5 W	5 W	4+	many	179		1	Died 32 D after onset

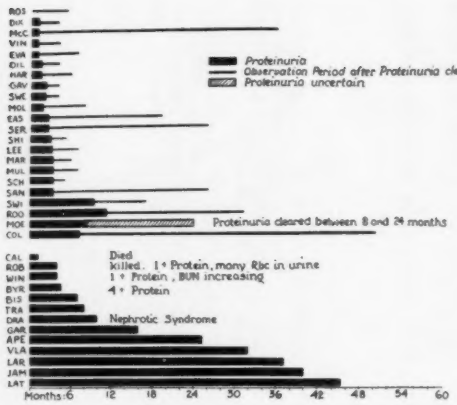


CHART 1. DURATION OF PROTEINURIA AND OBSERVATION PERIOD IN PATIENTS WITH ACUTE GLOMERULONEPHRITIS. There are two errors in this chart: Patient Eik was accidentally omitted from the first group and Col's proteinuria persisted for 26 months, not 8 months as indicated on the chart.

tococcal infection in each of these 4 patients. Evidence that the streptococcus was Type 12 was obtained in 11 of the 33 patients in whom sera were obtained for Type 12 antibodies.

Only patients from whom at least one adequate renal biopsy was obtained within 6 months of the onset of nephritis are included in the present analysis. Pertinent data concerning the patients, their disease and the evidence for preceding streptococcal infection are summarized in Tables I and II. Nineteen of the 36 patients were studied at the United States Naval Hospital, Great Lakes, Ill. The majority of these were transferred to this hospital from elsewhere in the United States or, in several instances, from overseas bases. The remaining patients were studied in hospitals affiliated with Northwestern University Medical School. Evidence of previous diffuse renal disease was not obtained in any of the patients. Records of previously normal urine examinations were available in many (Table I, column 4). Further, the latent period between precipitating infection and the onset of symptoms or signs of acute nephritis exceeded 5 days in 29 of the 33 patients in whom the latent period could be estimated. Latent periods of 5 to 28 days are characteristic of the initial attack of acute poststreptococcal glomerulonephritis, in contrast to 1 to 5 days of the exacerbation in chronic glomerulonephritis (26).

All but 4 of the patients were males, and all but 7 were less than 40 years of age; 22 were males between the ages of 17 and 25 years; 1 was a 10 year old female. We wish to emphasize that the majority of patients discussed in this report represent instances of sporadic acute glomerulonephritis in the adult. None of the patients contracted the disease under the epidemic conditions so ably described by Rammelkamp (27) and Stetson and colleagues (28) although 1 patient was the second of 4 members of a family of 7 who developed acute nephritis associated with Type 12 streptococcal infections.

Excluded from the present analysis were patients with clinical acute glomerulonephritis in whom we failed to obtain evidence of hemolytic streptococcal infection. This excluded group comprised 10 patients with nonstreptococcal acute nephritis previously reported (22) and 3 additional patients observed since that report. Also excluded were 4 patients who were seen too late to determine with certainty whether or not the associated infection had been of streptococcal origin or in whom the first adequate renal biopsy was obtained more than 6 months after onset. Three patients with poststreptococcal exacerbations in chronic glomerulonephritis likewise were excluded from the present considerations, as was a considerable number of patients studied only during the chronic phase of glomerulonephritis.

Clinical observations. All patients were followed by routine clinical and laboratory techniques. Positive and negative physical findings were recorded. Serial urinalyses and serial measurements of blood urea nitrogen were performed in all patients. Other nitrogenous wastes and electrolytes were determined when indicated. Most had one or more urea clearance determinations. Almost all had urine concentration tests and phenolsulphonphthalein (PSP) excretion tests.

The majority had moderately severe clinical acute glomerulonephritis (see Table II), although the diagnosis in 6 patients who had no renal symptoms was established only by routine examination of the urine following admission to the hospital for infection. Many presented gross hematuria, others malaise, headaches, edema or dyspnea. All but 1 of the patients had proteinuria and all had hematuria, which was gross in 18 of the 36; another 13 patients had many red blood cells in the urinary sediment. The duration of proteinuria and observation periods is shown in Chart 1.

Bacteriologic and immunologic studies. Throat cultures obtained more than 4 weeks after onset of infection are listed in Table I as "not done," as were cultures obtained only after the beginning of antibiotic therapy. Titers of serum antibodies against hemolytic streptococci were measured in all patients. Antistreptolysin O titers were measured by the method of Rantz and Randall (29), antistreptokinase by the method of Christensen (30) and antihyaluronidase by the method of Harris and Harris (31). Variations in antibody titers less than two tubes were not considered significant. Antibodies specific against Type 12 hemolytic streptococci were measured by Stollerman, Kantor and Gordon's modification (32) of the bactericidal method of Todd (33). The blood remaining in the biopsy needle was cultured in each instance in thioglycollate medium. All such cultures were sterile.

Clinical criteria for healing and chronicity. Permanent disappearance of proteinuria (at least 4 protein-free urines obtained over several weeks) was taken as evidence of clinical healing. Many more urines over considerably longer periods of time were obtained in the

TABLE III

Data relating to renal biopsies *

Patient	Approximate time of biopsy: After onset	Approximate time of biopsy: Disappearance, proteinuria: Before	Approximate time of biopsy: Disappearance, proteinuria: After	Findings at time of biopsy: Urine: Prot.	Findings at time of biopsy: Urine: RBC	Findings at time of biopsy: BUN	Findings at time of biopsy: Blood pressure	Biopsy: Length	Biopsy: Glomeruli
	days	*days*				*mg %*	*mm Hg*	*mm*	*no.*
				Healed					
Swi	32	256		trace	occ.	12	140/60	8	8
	79	209		trace	occ.		120/60	6	5
Dil	82		27	0	occ.	14	120/80	7	22
Ros	17	no proteinuria		0	rare	15	120/70	3.5	14
Sch	132	7		trace	few	15	122/66	8	36
Shi	120		2	0	0	12	120/70	7	17
Gav	68		23	0	0	18	140/80	2	13
Swe	72	4		trace	few	15	120/85	6	17
Eas	66	24		1+	few	20	128/84	8	11
	115		25	0	0	20	125/80	9	34
Ser	30	60		2+	many	17	136/80	5	6
	80	10		trace	occ.	20	124/80	10	15
	156		66	0	0	14	138/80	5	5
	775		685	0	0	16	126/88	5	5
Mol	22	58		2+	many	19	135/62	3	10
	114		34	1+†	many	15	120/60	8.5	34
	205		126	0	0	8	125/60	6.5	9
Dix	32		3	0	0	17	128/86	8	25
Mul	11	49		trace	occ.	18	140/80	3	10
Moe	170			trace	occ.	18	125/80	9	19
Vin	15	10		1+	few	25	140/96	8.5	5
Har	39	21		trace	many	25	148/96	19	15
Roo	47	306		1+	rare	20	120/70	5	10
	602		249	0	0	22	130/80	5.5	11
Col	66	732		2+	occ.	14	142/100	8	12
	197	603		trace	0	15	128/78	6	12
	1,356		556	0	0	15	115/82	7	9
Eva	144		61	0	many	15	130/80	2.5	13
Eik	24	28		1+	occ.	19	126/90	15	10
San	33	89		1+	occ.	34	156/90	10	7
	73	63		trace	occ.	12	110/52	4	14
	149		61	0	0	19	110/52	4	1
McC	20	2		trace	rare	17	180/90	5	15
Lee	31	74		2+	many	51	126/86	10	16
	158		14	0	0	17	120/80	5	9
Mar	27	39		2+	many	28	148/80	3	5
				Not healed at last follow-up					
Dra	122			4+	many	15	130/84	5	19
Byr	14			2+	many	17	124/80	6.5	20
	115			2+	many	14	120/80	6	21
Lar	79			0‡	occ.	11	145/90	4	35
Rob	97			1+	many	24	120/80	6	6
Bis	146			2+	many	20	110/80	4	7
Jam	28			trace	few	19	134/90	8	9
	1,013			2+	0	14	159/90	10	37
	1,234			2+	occ.	16	140/90	7	12
Win	61			1+	occ.	15	112/70	12	13
Gar	129			3+	few	25	200/120	7	13
Lat	122			1+	few	25	135/95	11	25
Ape	22			3+	many	52	180/100	8	15
	69			2+	occ.	38	140/100	7	14
	307			1+	occ.	18	178/92	7	1
Vla	82			1+	many	45	138/100	6	10
	162			1+	occ.	27	120/72	2	5
Tra	71			1+	occ.	13	135/80	7	15
	186			1+	few	15	130/80	8	11
				Died					
Cal	30			4+	many	179	178/116	10	16

* Abbreviations as in previous tables.
† Urine day before biopsy contained 1+ protein. Many urines before and after biopsy contained no protein.
‡ Previous and subsequent urines contained protein.

TABLE IV

Histologic findings in 36 patients with proved post-streptococcal acute glomerulonephritis

Patient	Case history no.	Biopsy, after onset	Glomeruli	Glomerular changes: Hyalinized glomeruli	Diseased glomeruli	Hypercellularity*	Endothelial prolif.†	Leukocytes per glom.‡	Lobular necroses or scars§	Crescents‖	Adhesions to: Crescents	Adhesions to: Other	Interstitial fibrosis¶: Recent	Interstitial fibrosis¶: Healed	Disease of vessels: Arteries	Disease of vessels: Arterioles	Tubules: RBC or heme casts
		days	*no.*	*no.*					*no.*	*no.*	*no.*						
								Healed									
Swi	9	32	8	0	0	0	0	0/0–1	0	0	0	0	0	0	0	0	0
		79	5	0	0	0	0	0/0–1	0	0	0	0	0	2+	0	0	0
Dil		82	22	0	all	1+	1+, L.S.	0/1	0	1	0	0	0	1+	0	0	0
Ros		17	14	0	0	0	0	0/0	0	0	0	0	0	0	0	0	0
Sch		132	36	0	all	2+	2+, L.S.	0/0–1	0	0	0	0	0	0	0	0	0
Shi	10	120	17	1	a few	±	±, L.S.	0/0	1S	0	0	1	0	1+	0	0	0
Gav		68	13	0	all	2+	2+, L.S.	0/0–1	0	0	0	0	0	1+	0	0	0
Swe		72	17	0	all	1+	1+, L.S.	0/0–1	0	0	0	0	0	0	0	0	0
Eas		66	11	0	all	2+	2+, L.S.	0/0–1	0	0	0	0	0	1+	0	0	few
		115	34	0	all	2+	2+, L.S.	0/0–1	0	0	0	0	0	1+	0	1+	0
Ser	3	30	6	0	all	3+	2+, Dif.	2/13	0	0	0	0	0	0	0	0	0
		80	15	0	all	2+	2+, L.S.	0/1–6	0	0	0	0	1+	0	0	0	0
		156	5	0	all	2+	2+, L.S.	0/2	0	0	0	0	0	1+	0	0	few
		775	5	0	0	±	±	0/0–1	0	0	0	0	0	0	0	0	0
Mol	2	22	10	0	all	3+	2+, Dif.	10/12	0	0	0	0	1+	0	0	0	many
		114	34	0	all	2+	2+, L.S.	0/1–2	3S	0	0	3	0	2+	0	0	few
		205	9	1	all	1+	1+, L.S.	0/1–2	0	0	0	0	0	2+	0	0	0
Dix		32	25	0	all	2+	1+, L.S.	0/4	0	0	0	1	0	0	0	0	0
Mul		11	10	0	all	2+	1+, L.S.	1–3/7–24	0	0	0	0	2+	0	0	1+	few
Moe		170	19	0	all	2+	2+, L.S.	0/0–1	0	0	0	1	0	2+	0	0	0
Vin		15	5	0	all	3+	2+, Dif.	0/5–22	0	0	0	0	1+	0	0	1+	rare
Har		39	15	1	all	2+	2+, L.S.	0/1–6	0	0	0	0	2+	0	1+	1+	many
Roo		47	10	0	all	1+	1+, L.S.	0/1–2	0	0	0	0	0	1+	0	2+	few
		602	11	3	0	0	0	0/0–1	0	0	0	0	0	2+	0	2+	few

* Hypercellularity = total increase in number of cells within glomerulus including both endothelial cells and leukocytes. Almost total obliteration of capillary loops by cells equals 4+ hypercellularity.

† L.S. = lobular stalks; Dif. = diffuse.

‡ Leukocytes per glomerulus = eosinophiles/polymorphonuclear neutrophiles.

§ N = lobular necrosis; S = lobular scar.

‖ hd = healed; hg = healing.

¶ Healed: 0 = no fibrosis; 1+ = occasional tiny periglomerular foci; 2+ = larger cortical scars; 3+ = 50% or more scarring in cortex; 4+ = diffuse fibrosis. Recent: edema with some inflammatory cells and fibrosis on same scale as above.

TABLE IV—(*Continued*)

Patient	Case history no.	Biopsy, after onset	Glomeruli	Glomerular changes: Hyalinized glomeruli	Diseased glomeruli	Hypercellularity*	Endothelial prolif.†	Leukocytes per glom.‡	Lobular necroses or scars§	Crescents‖	Adhesions to: Crescents	Adhesions to: Other	Interstitial fibrosis¶: Recent	Interstitial fibrosis¶: Healed	Disease of vessels: Arteries	Disease of vessels: Arterioles	Tubules: RBC or heme casts
		days	*no.*	*no.*					*no.*	*no.*	*no.*						
								Healed									
Col	8	66	12	1	all	2+	2+, L.S.	0/2–3	2S	4	3	1	2+	2+	1+	0	many
		197	12	1	all	2+	2+, L.S.	0/1–2	3S	1 hd 3 hg	3	4	0	3+	1+	0	few
		1,356	9	3	all	1+	1+, L.S.	0/1–2	0	0	0	2	0	1+	0	2+	0
Eva	1	144	13	0	all	2+	2+, L.S.	0/0–1	0	0	0	0	1+	0	0	0	0
Eik		24	10	0	all	2+	1+, L.S.	1/5	0	0	0	0	0	0	1+	2+	few
San	4	33	7	0	all	4+	3+, Dif.	3/9	0	0	0	0	0	0	0	0	0
		73	14	0	all	2+	2+, L.S.	0/2–3	0	0	0	0	0	0	0	0	0
		149	1	0	all	2+	2+, L.S.	0/0–1	0	0	0	0	0	0	0	0	0
McC		20	15	11	all	2+	1+, Dif.	0/11	0	1	0	0	0	3+	4+	3+	0
Lee		31	16	0	all	3+	1+, L.S.	0/3–9	0	5	5	0	2+	2+	0	2+	many
		158	9	0	all	1+	1+, L.S.	0/0–1	2S	0	0	2	0	2+	1+	1+	rare
Mar		27	5	0	all	3+	2+, Dif.	1/14–30	0	0	0	0	2+	0	0	1+	0
								Not healed at last follow-up									
Dra		122	19	0	all	1+	1+, L.S.	0/0–1	5S	6 hg	6	2	0	3+	0	0	many
Byr		14	20	0	all	2–4+	2–4+, Dif.	1/2	0	3	2	0	1+	0	0	0	few
		115	21	0	all	1+	1+, L.S.	0/0–1	0	0	0	0	1+	0	0	0	0
Lar		79	35	0	all	3+	3+, L.S.	0/0–1	0	0	0	0	0	0	0	0	0
Rob		97	6	0	all	3+	3+, L. S.	0/1	4N	4 hg	4	2	0	3+	0	0	many
Bis		146	7	0	all	3+	3+, L.S.	0/0–1	0	0	0	2	0	2+	0	0	0
Jam		28	9	0	0	0	0	0/0	0	0	0	0	2+	2+	0	0	0
		1,013	37	0	few	1+	1+, L.S.	0/0	2S	0	0	0	0	1+	0	0	0
		1,234	12	0	0	0	0	0/0	0	0	0	0	0	0	0	1+	0
Win		61	13	0	all	3+	3+, L.S.	0/2	3N	3 hg	3	0	2+	2+	1+	0	few
Gar		129	13	4	all	3+	3+, L.S.	0/3–5	11N	3	3	8	0	3+	1+	0	few
Lat		122	25	0	all	3+	3+, L.S.	0/0–1	0	0	0	0	0	1+	0	0	few
Ape	6	22	15	0	all	4+	1+, L.S.	1/15–31	2N	4	4	2	4+	0	1+	±	many
		69	14	0	all	1+	1+, L.S.	0/0–1	2S	2 hg	2	4	1+	4+	0	1+	many
		307	1	0	all	2+	2+, L.S.	0/0	0	0	0	0	0	3+	0	0	few
Vla		82	10	4	all	1+	1+, L.S.	0/1–2	7S	6 hg	6	1	2+	3+	0	0	many
		162	5	1	all	1+	1+, L.S.	0/0	2S	2 hg	2	7	0	2–3+	0	0	0
Tra	5	71	15	0	all	3+	3+, L.S.	0/2	2S	1	1	2	3+	2+	0	0	many
		186	11	0	all	2+	2+, L.S.	0/1	3S	2	2	3	1+	2+	0	0	few
								Died									
Cal	7	30	16	0	all	4+	0	3/70	16N	16	16	16	4+	0	0	0	many

TABLE V

Types of glomerular lesions observed in relation to time of biopsy after onset and to clinical outcome

Days after onset	Type of glomerular lesion							
	Presumed focal		Primarily proliferative		Primarily proliferative with exudation or damage		Primarily exudative	
	Patient*	Outcome	Patient	Outcome	Patient	Outcome	Patient	Outcome
11					Mul	Healed		
14			Byr	Chronic				
15					Vin	Healed		
17	Ros	Healed						
20			McC	Healed				
22			Mol	Healed			Ape	Chronic
24			Eik	Healed				
27					Mar	Healed		
28	Jam†	Chronic						
30			Ser	Healed			Cal	Died
31							Lee	Healed
32	Swi	Healed	Dix	Healed				
33			San	Healed				
39			Har	Healed				
47			Roo	Healed				
61					Win	Chronic		
66			Eas	Healed			Col	Healed‡
68			Gav	Healed			(Ape	Chronic)
71			Swe	Healed	Tra	Chronic		
73			(San	Healed)				
79	(Swi	Healed)	Lar	Chronic				
80			(Ser	Healed)				
82			Dil	Healed			Vla	Chronic
97					Rob	Chronic		
114			(Mol	Healed)				
115			(Byr	Chronic)				
115			(Eas	Healed)				
120	Shi	Healed						
122			Lat	Chronic			Dra	Chronic
129					Gar	Chronic		
132			Sch	Healed				
142			Eva	Healed				
146					Bis	Chronic		
149			(San	Healed)				
156			(Ser	Healed)				
158							(Lee	Healed)
162							(Vla	Chronic)
170			Moe	Healed				
186					(Tra	Chronic)		
197							(Col	Healed‡)
205			(Mol	Healed)				
307							(Ape	Chronic)

More than one year after onset

	Glomerular lesion	Name	Outcome
602	Normal, hyalinized	(Roo	Healed)
775	Minimal proliferative	(Ser	Healed)
1,013	Normal	(Jam†	Chronic)
1,234	Normal	(Jam†	Chronic)
1,356	Miminal proliferative with glomerular damage	(Col	Healed)

* Parentheses indicate that patient had previous biopsy.
† Interstitial reaction as well as presumed focal glomerulitis.
‡ Proteinuria disappeared late (26 months after onset).

majority of patients listed as healed in the tables. Of the 23 patients who are considered to have achieved clinical cure, proteinuria persisted more than 6 months in only 4, lasting 26 months in 1 patient (Chart 1). This long duration of proteinuria is distinctly unusual among patients whose acute glomerulonephritis heals. Three patients (Dil, Eva, Mul) still had microscopic hematuria at the time of last study, but nevertheless are considered to have healed clinically. Microscopic hematuria not infrequently persists some months longer than proteinuria

in patients who eventually achieve permanent clinical healing (34).

Persistence of proteinuria for a year or more after the acute onset is considered to be reasonably good evidence for chronicity of glomerulonephritis (35) although we recognize that exceptions may occur (e.g., Patient Col). Of the 13 patients who are listed as "not healed at the time of last examination," proteinuria was documented for a year or more in 6. One patient died of hyperkalemia 32 days after onset, and undoubtedly his renal disease would have become chronic had he survived the acute phase.

The estimated time, after the onset of clinical acute glomerulonephritis, at which biopsies were obtained is given in Table III along with urine, blood urea nitrogen, and blood pressure observations at the time of each biopsy. No biopsy containing less than 5 glomeruli was included unless it was one of a series from the same patient that included one or more adequate biopsies.

Biopsy and histologic techniques. Renal biopsies were obtained by Kark and Muehrcke's percutaneous technique (36). No complications developed, except for pain at the time of actual biopsy in less than 5 per cent of the attempts. Specimens were fixed for 3 hours in Helly's solution, washed 3 hours in running water and embedded in paraffin. Sections cut at 2 and 6 μ were stained with hematoxylin and eosin (H & E), the periodic acid-Schiff (PAS) method with a hematoxylin counterstain, and Heidenhain's modification of Mallory's connective tissue stain (CT). The PAS methenamine stain of Jones (37), iron (38), hemoglobin (39), and Weigert-van Gieson stains were used when indicated. Tissue from the biopsy specimen was prepared for electron microscopy in 16 patients. For this purpose, 0.5 to 1 mm cylinders, cut from a portion of the biopsy that was believed to be cortex, were fixed in buffered osmium tetroxide which contained sucrose and dextran (40). The tissue was embedded in a 1:1 mixture of butyl and methyl methacrylate and then sectioned at 0.5 μ and at 0.025 μ with a Porter-Blum microtome and a glass knife. The 0.5-μ sections were stained with polychromatic methylene blue and studied by light microscopy. The thin sections were studied at 100 kv in an RCA EMU3C electron microscope.

OBSERVATIONS

I. Histopathologic changes

Data concerning the renal biopsies and the status of the patients at time of biopsy are summarized in Table III. The duration of proteinuria and of the follow-up periods are shown in Chart 1.

A summary of the histologic features of each biopsy of the 36 patients is given in Table IV. The primary changes were glomerular. Several types of glomerular lesions were noted; endothelial cell proliferation and glomerular exudation were the most characteristic. In the early stages

TABLE VI

Classification and clinical outcome of 26 patients with primarily proliferative glomerulonephritis

Classified by endothelial proliferation*		No. of patients	Clinical outcome: Healed	Clinical outcome: Chronic
Early†:	Mild (1+)	4	Dix, Eik, McC, Mul	0
	Moderate (2+)	5	Har, Mar, Mol, Ser, Vin	0
	Marked (3–4+)	2	San	Byr
Late:	Mild (1+)	3	Dil, Roo, Swe	0
	Moderate (2+)	5	Eas, Eva, Gav, Moe, Sch	0
	Marked (3–4+)	7	O	Bis, Gar, Lar, Lat, Rob, Tra, Win
Classified by associated exudation‡				
Early†:	Focal§	3	Mar, Mul, Vin	0
	Mild	4	Dix, Eik, Har	Byr
	Moderate	4	McC, Mol, San, Ser	0
Late:	None	9	Dil, Eas, Eva, Gav, Roo, Sch, Swe	Lar, Lat
	"Glomerular damage"‖	6	Moe	Bis, Gav, Rob, Tra, Win

* Endothelial proliferation graded on the 0–4+ scale. In the late group we assume that the degree of lobular stalk hypercellularity exhibited is a reflection of the degree of proliferation that would have been present had the patient been biopsied shortly after the onset.

† Early: first biopsy obtained within 6 weeks of onset.

‡ Mild: 2–9 inflammatory cells per glomerulus; moderate: 10–20 inflammatory cells per glomerulus.

§ Focal exudation: clogging of a glomerular lobule with inflammatory cells with or without necrosis, capillary thrombosis or fibrin deposition.

‖ Glomerular damage: lobular scarring, crescents and adhesions between capillary loops and Bowman's capsule.

TABLE VII

Classification and clinical outcome of 6 patients with primarily exudative glomerulonephritis

Classified by degree of exudation*		No. of patients	Clinical outcome: Healed	Clinical outcome: Chronic
Early†:	Mild	1	Lee	0
	Moderate	1	0	Ape
	Marked	1	0	Cal (died)
Late:	Glomerular damage	3	Col	Dra, Vla
Classified by associated endothelial proliferation				
Early†:	None	1	0	Cal (died)
	Mild (1+)	2	Lee	Ape
Late:	Mild (1+)	2	0	Dra, Vla
	Moderate (2+)	1	Col	0

* Degree of exudation based on number of inflammatory cells in glomeruli, but all patients also had crescents, adhesions and other glomerular damage which are difficult to quantitate.

† First biopsy obtained within 6 weeks of onset.

endothelial proliferation generally was diffusely distributed within capillary loops. Later the hypercellularity was more focal and located in the "stalks" of each glomerular lobule. The exudative reaction, the acute stages of which rarely extended beyond 6 weeks, was characterized by an increased number of polymorphonuclear leukocytes within the glomerular capillary loops. Diffuse and focal distributions of the leukocytes were noted with almost equal frequency. Other evidence of glomerular injury included lobular necroses or scars, epithelial cell crescents and adhesions between glomerular lobules and epithelial cell crescents or Bowman's capsule; capillary loop thrombi were seen in 2 patients. Such evidences of glomerular injury with rare exceptions exhibited focal distribution. In several parts of the text and in several tables these lesions have been grouped together, for convenience, under the term *glomerular damage*.

The patients were grouped by an arbitrary system based on the predominant glomerular lesions noted in their renal biopsies (Table V). Three patients were classified as *presumed focal glomerulitis*, 1 as *presumed focal glomerulitis and interstitial reaction*, 18 as *primarily proliferative*, 8 as *primarily proliferative with glomerular damage*, and 6 as *primarily exudative*. Leukocytic infiltration, the most characteristic feature of the exudative process, was rare after 6 weeks, and, when present after 6 weeks, was minimal. However, of the 25 patients first biopsied more than 6 weeks after onset, 3 (Col, Vla, Dra) were classified arbitrarily as primarily exudative on the basis of prominent glomerular scarring and only mild proliferative changes. The glomerular changes in these 3 patients were very similar to those noted in Ape and Lee, whose earlier biopsies revealed unequivocal exudative reactions. Proliferative changes were more marked, and scars, adhesions and crescents less marked and more focal, in the 8 patients classified as primarily proliferative with glomerular damage. Five biopsies were obtained from 4 of the patients more than 1 year after onset. The classifications of the 36 patients are summarized in several different ways in Tables V, VI and VII. Reference to these tables will assist in orienting the reader in several parts of the text.

1. Acute proliferative glomerulonephritis. The predominant glomerular lesion in 26 patients (Tables IV–VI) was an increased number of endothelial cells within the glomerular capillary loops of all unhyalinized glomeruli (Figures 1–12). This glomerular response, which corresponds to classic acute proliferative glomerulonephritis, was the most characteristic feature of our patients with proved post-streptococcal acute glomerulonephritis. The extent of the endothelial proliferation was variable, although in each of these 26 patients all glomeruli were involved to some degree.

Eleven patients were biopsied in the early phase of the proliferative response (first 6 weeks after onset). Three additional patients (Mar, Mul, Vin) had diffuse endothelial proliferation complicated by considerable focal exudation. Most of the new endothelial cells had vesicular nuclei which appeared larger and paler than those of normal endothelial cells (Figures 2, 4, 9, 12). Their cytoplasm was pale, stained lightly with both eosin and azocarmine, and was only slightly positive to the PAS reaction. Small hyaline fibers, which stained blue with Heidenhain's stain, or brownish black with the PAS-methenamine stain, were prominent in the cytoplasm of the endothelial cells, especially in areas of marked hypercellularity (Figures 7, 12).

The degree of hypercellularity varied considerably from patient to patient, ranging from about 1.5 to 3.5 times normal. The mildest proliferative changes were noted in Mul, Dix, Eik and McC

(Figures 10, 11), and should be contrasted with the more marked proliferation exhibited in some glomeruli in Byr (Figure 5) and in almost all of the glomeruli of San (Figures 1, 2, 12). The new cells were usually found in small clusters in a central position in the lobules of the glomerulus in the mild cases, but completely filled many of the lobules in patients with marked proliferative response. The capillary loops seemed to be completely occluded in the areas of marked proliferation (Figures 1, 2, 5, 12) but were usually widely patent in the mild cases, particularly those at the periphery of the lobules (Figures 10, 11).

The degree of hypercellularity also varied among different glomeruli in the same biopsy. This appeared clearly in Byr, some glomeruli showing a marked proliferative response, and others much less severe changes (Figure 5). In addition, different lobules within a single glomerulus frequently varied considerably in degree of involvement. Ser, Byr and Mol all showed this variation (Figures 3, 6, 9).

Careful search of thin sections of glomeruli in the patients in the early phases of acute proliferative glomerulonephritis revealed mitotic figures in Byr and San. Some of the mitoses are shown in Figures 1, 2 and 8. The mitoses always occurred in cells that were located within and attached to the glomerular capillary basement membrane (endothelial cells). San had as many as three mitoses per 2μ section of glomerulus.

All 11 patients with proliferative acute glomerulonephritis studied within 6 weeks of onset exhibited some increase in the number of polymorphonuclear neutrophiles within capillary loops, although the increase was minimal (5 or less per glomerulus) in 4 patients. In all instances the number of intraglomerular leukocytes noted in the proliferative group was less than that noted during the acute phase of the exudative response, when as many as 75 polymorphonuclear neutrophiles were observed within a single glomerulus (*vide infra*). Eosinophilic polymorphonuclear leukocytes were noted in addition to the neutrophiles in 5 patients in the proliferative group. The significance of the eosinophilia is not known. Eosinophiles were not noted in later biopsies from these patients, nor was significant eosinophilia in the circulating blood demonstrated in any. The leukocytes were diffusely distributed throughout the glomerular capillary loops in 7 patients. In 3 (Mar, Mul, Vin) the exudative process was focal in distribution. For instance, several lobules in Vin's biopsy contained as many as 15 neutrophiles. Other entire glomeruli contained only a third of this number of leukocytes. Similar findings are illustrated in the biopsy shown in Figure 29 (Mar), where the lobule at 8 o'clock contains 22 leukocytes.

Evidences of glomerular damage (lobular necrosis or scars, crescents, adhesions or capillary thrombi) were noted in 5 of the 11 patients in the early proliferative group. More than 20 per cent of the glomeruli were involved in only 2 of these. One (Mar) had focal exudation. The other (McC) was a 59 year old male who had significant arterio- and arteriolar nephrosclerosis that probably were responsible for the 11 hyalinized glomeruli among the total of 15 in the biopsy. Two of the patients (Mar, Mul), biopsied within 4 weeks of onset, had occasional thrombi in capillary loops (Figure 29).

Few abnormalities were noted in the glomerular epithelial cells in the early primarily proliferative group except in 2 patients (Byr, McC) in whom crescents were present (Figure 8). One crescent in Byr contained a mitotic figure. The cytoplasm of some of the epithelial cells covering the capillary loops contained hyaline droplets that stained with azocarmine and were positive to the PAS reaction (Figures 6, 8). Similar droplets were common in the cytoplasm of the proximal tubular cells in these patients.

The basement membranes of the peripheral glomerular capillary loops of the unhyalinized glomeruli were normal in all patients in the early primarily proliferative group. Small foci of periglomerular edema and early interstitial fibrosis were noted in 8 of the 11 patients in the group. These foci usually contained a few atrophic tubules. Patient McC had rather severe interstitial fibrosis. Some of this was of recent origin, but much apparently was the result of long-standing vascular disease. The lack of over-all acute disruption of cortical architecture in this group was striking, especially when compared with the findings in the primarily exudative group (Patients Ape and Cal).

The histologic changes in subsiding acute proliferative glomerulonephritis of moderate or marked

severity (from 6 weeks to 7 months after onset) are illustrated by subsequent biopsies in 4 of the proliferative cases (Ser, Byr, San, Mol). Glomerular hypercellularity was still present, but the pattern had shifted from a generally diffuse to a more focal distribution. The hypercellularity was now the result of a collection of cells in the stalks of each glomerular lobule (Figures 14–26). The nuclei of the cells in the hypercellular foci were more basophilic and less vesicular than the average endothelial cell nucleus noted in the acute stage. Their cytoplasm was eosinophilic, positive to the PAS reaction, and stained blue with aniline blue. Cellular borders were not present, and small, linear, irregular hyaline intracytoplasmic fibers that stained a bright refractile blue with aniline blue, with much the same intensity and coloration as basement membrane, were readily seen in thin sections (Figure 13). These fibers had some of the staining reactions of collagen. However, they did not stain with silver except after prior oxidation with periodic acid (Figure 26). Also, these fibers did not show the typical periodicity of collagen in electron micrographs of the biopsy obtained 215 days after onset in Mol (Figures 27, 28).

Similar lobular stalk hypercellularity was observed in the glomeruli of the patients in the proliferative group who were biopsied for the first time 6 weeks or more after onset. The degree of hypercellularity varied considerably (Figures 30–37). Some glomeruli were more hypercellular than others in the same biopsy, and frequently some lobules within a single glomerulus were more hypercellular than others (Figure 34). The actual number of cells within the lobular stalks probably was directly related to the degree of proliferation present during the acute phase.

Exudative changes, particularly those of the focal type, that are of sufficient severity to lead to scarring (e.g., Mar, Vin, Mul) or are manifest later by lobular scarring, are a prominent variable relating to the severity of any given attack of glomerulonephritis. This generalization can best be demonstrated by contrasting the course of Patient San (Case 4, Figures 1, 12, 21, 22), who had relatively pure marked proliferative glomerulonephritis without glomerular damage and interstitial scarring, with the course of Patient Tra (Case 5, Figures 63, 64), who exhibited signs of marked initial proliferation—namely, striking lobular stalk hypercellularity, plus lobular scars, adhesions and severe interstitial fibrosis. Tra's histopathologic findings apparently are the result of an initial mixed proliferative and exudative response. Very similar changes were shown by Rob (Figures 53–55), Gar and Win. They also are believed to be the result of an initial mixed proliferative and exudative response.

Only 2 patients (Ser, Gar) showed signs of active acute glomerular inflammation more than 6 weeks after onset. Glomeruli in a biopsy obtained 129 days after onset in Gar contained 3 to 5 polymorphonuclear leukocytes per glomerulus in the 2μ sections. Occasional deposits of fibrinoid in the capillary loops and lobular stalks also were present. A few capillary basement membranes in most glomeruli were thickened, the only instance of thickened basement membranes in significant numbers in our entire series. Occasional glomerular endothelial cells with abundant finely vacuolated cytoplasm, presumably filled with fat droplets, also were noted. The cytoplasm of some of the proximal tubules contained multiple small cytoplasmic vacuoles which were likewise presumably fat. Cases 1 through 5 in the protocols illustrate the proliferative group.

Biopsies were obtained more than a year after the onset in only 2 patients in the proliferative group. Roo, who had mild hypercellularity initially, showed no recognizable signs of antecedent glomerular disease (Figure 38) in a biopsy taken 602 days after the onset and 249 days after disappearance of proteinuria (Figure 39). Ser, who had moderate hypercellularity initially, showed very minimal lobular stalk thickening without much hypercellularity in 1 of 2 glomeruli obtained in a biopsy 26 months after the onset (Figure 18).

2. Primarily exudative glomerulonephritis. All 3 patients, so classified and biopsied within 6 weeks of onset, showed marked increases in the number of polymorphonuclear neutrophiles within the glomeruli. Endothelial cell proliferation, although present, in Ape and Lee was not a prominent feature (Figure 41). No evidence of endothelial proliferation was noted in Cal. The diagnosis of exudative glomerulonephritis can be made objectively only during the early phases of the dis-

ease. After the leukocytes have disappeared, usually 50 to 60 days after onset, it is impossible to be certain that the initial disease was primarily exudative in character.

Ape, Lee and Cal, in addition to infiltration of the glomeruli with polymorphonuclear leukocytes also exhibited glomeruli with adhesions (Figures 42, 43, 45, 46), lobular necrosis (Figures 42, 43), and crescents (Figures 42, 45, 46). The cortical tissue was edematous (Figures 40, 45). Many proximal tubules were lined by regenerating or flattened epithelium. Later biopsies from Ape showed diffuse and marked interstitial fibrosis (Figures 48, 49). The second biopsy obtained 69 days after onset had 0 to 1 neutrophiles per 2μ section of glomerulus and very slight lobular stalk hypercellularity (Figure 49). Some glomeruli also showed lobular scars and adhesions (Figure 50). Her third biopsy, 307 days after onset, contained a single glomerulus which showed severe disease. It was adjacent to an area of marked juxtamedullary cortical inflammation and fibrosis. Lee's second biopsy, 158 days after onset, showed glomerular lobular scars, slight lobular stalk hypercellularity and considerable focal interstitial fibrosis.

Few changes in glomerular ultrastructure were observed by electron microscopy in the portions of glomeruli lacking crescents in the biopsy on Cal. The basement membranes and the foot processes of the epithelial cells over the basement membranes were normal (Figure 47). Endothelial cell proliferation was absent. The cytoplasm of the endothelial cells formed a thicker layer than is usually observed in glomeruli, and numerous polymorphonuclear neutrophiles were present in the capillary loops, apparently adherent to the cytoplasm of the endothelial cells. Case histories of Ape and Cal are given in the protocols (Cases 6 and 7) and should be contrasted with Cases 1–5.

Patients Col (Case 8, Figures 51, 52, 56, 57), Vla (Figures 58–62), and Dra were considered to be examples of primarily exudative glomerulonephritis (Tables V, VII). The first biopsies in these patients were not obtained until 2 to 4 months after onset, at a time when the glomerular leukocytic infiltration would be expected to have disappeared and the acute exudative lesions to be healed or healing. Occasional glomeruli in each patient showed mild lobular stalk hypercellularity, indicating that some initial proliferative disease had been present. The extensive glomerular scarring and interstitial fibrosis in the absence of marked proliferation, and the similarity of the biopsies to the changes noted in late biopsies on Ape and Lee, led to our arbitrary decision to place these 3 patients in the exudative group.

3. Presumed focal glomerulitis. Patients Ros, Swi (Case 9 in protocols) and Shi (Case 10) comprise this group (Tables IV, V). Focal and very slight lobular stalk hypercellularity was present in most glomeruli in Shi's biopsy, obtained 120 days after onset (Figure 65). One glomerulus showed a lobular scar adherent to a small healing crescent (Figure 66). Biopsies obtained in all other patients with subsiding proliferative glomerulonephritis, at about the same time after onset as in Shi, revealed diffuse glomerular involvement. Although unusual in post-streptococcal glomerulonephritis, focal glomerular lesions similar to Shi's may have been present in Ros and Swi, but were not included in the small samples of glomeruli obtained by biopsy. This situation has been reasonably well established in an outbreak of nonstreptococcal acute nephritis (*22*). Significant glomerular lesions were not noted in either Ros or Swi, even though biopsies were obtained 32 and 17 days, respectively, after onset (Figures 67, 68). Alternatively, minor lesions not detectable by light microscopy could have accounted for the urinary abnormalities in Ros and Swi.

4. Interstitial reaction, presumed focal glomerulitis. Jam developed unequivocable laboratory evidence of acute glomerulonephritis 14 days after acute tonsillitis due to a Type 12 streptococcus. His history has been given in detail [Case 1 (*24*)]. During a long follow-up period (40 months) he exhibited persistent 1 + proteinuria and a labile hypertension. The first biopsy, obtained 28 days after onset, is illustrated in Figures 69 and 70. No overt glomerular lesions were present. The only unusual findings in the first biopsy were two tiny foci of interstitial edema, fibrosis, minimal chronic inflammatory cell infiltration and tubular atrophy. No organisms were identified in the sections, and cultures of the blood in the biopsy needle were sterile. Subsequent biopsies from this patient (Figure 71) up to 40 months after

onset of clinical disease showed no further changes, even though proteinuria persisted and mild hypertension developed. Electron micrographs of the last two biopsies showed normal glomerular ultrastructure. The last biopsy exhibited a few sclerotic arterioles. Whether the continued proteinuria resulted from a chronic glomerulonephritis not detectable on biopsy or from nephrosclerosis is not clear. The latter possibility seems the more likely in view of the absence of glomerular changes. Nevertheless, he is listed as chronic glomerulonephritis in the tables.

5. Tubular and interstitial changes. The cytoplasm of the proximal tubules of all patients who had proteinuria at the time of biopsy (Table III) contained some hyaline droplets that stained with azocarmine and were PAS-positive. Although erythrocytes were uncommon in Bowman's space, they were frequently noted in the lumens of the proximal tubules during the acute phase of the disease. Red blood cell casts, heme casts or granular casts containing erythrocytes were noted in 17 of the 36 patients, especially in the first 6 to 8 weeks of illness. Hyaline casts were uncommon except in the older patients or in patients who had considerable interstitial fibrosis.

Small scattered foci of interstitial fibrosis were noted in 11 of the 36 patients; moderate to severe interstitial fibrosis was noted in 17. With the exception of Jam, all patients with moderate to severe interstitial fibrosis also had evidence of moderate to marked proliferative or exudative glomerular disease. On the other hand, 4 patients (San, Lar, Lat, Byr) with marked proliferative disease had little or no interstitial reaction.

6. Vascular disease. No acute arteriolar disease with necrosis or fibrinoid was noted in any of the 36 patients; 11 did show eccentric subintimal hyaline deposits in the walls of arterioles. The arteriolar lesions were usually minimal in extent except in Roo, Eik and McC, who showed sufficient associated old interstitial fibrosis to be classified as arteriolar nephrosclerosis. The hyalinized glomeruli in McC probably were the result of the vascular disease. Curiously, the glomerulonephritis in these 3 patients was mild histologically, and healed by clinical standards. Fibrous arteriosclerosis was noted in one or more arteries in 8 patients, 3 of whom did not show associated arteriolar disease.

II. Relationship of biopsy findings to clinical course

The degree of proteinuria and hematuria noted at the onset of acute glomerulonephritis in all patients is given in Table II, along with the results of the last available urinalysis. Clinical healing occurred in the first 23 patients in Tables I and II and Chart 1. Proteinuria disappeared in 3 months or less in 14 patients, and in less than 6 months in 19; Col was the only patient in whom proteinuria is known to have disappeared later than 1 year after onset. Proteinuria in Patient Moe disappeared some time between 8 and 24 months after onset.

Among 13 patients who still had proteinuria at the time of last observation, the follow-up period exceeded 12 months in 6. That the remaining 7 patients have developed chronic glomerulonephritis, therefore, is not certain, although proteinuria has been documented for 5 months or more in all but 4. Nevertheless, certain observations suggest that several of the patients with relatively short follow-ups will, in fact, develop chronic glomerulonephritis. Heavy proteinuria was still present in Patients Dra and Byr at 10 and at 4.5 months, respectively. Patient Rob still had many erythrocytes in his urinary sediment and a 1+ proteinuria 4 months after onset. Unfortunately, he was killed in an accident 1 year after onset. Patient Cal died 32 days after onset, despite several dialyses on the artificial kidney. Had he survived the acute episode, he undoubtedly would have developed chronic glomerulonephritis or died from subacute disease.

For these reasons and for the purposes of the present paper, we will consider that all 13 patients who had proteinuria at the time of last examination are examples of the development of chronic glomerulonephritis following an attack of post-streptococcal acute glomerulonephritis. We recognize, however, that our judgment may be erroneous in several instances. Murphy and Schulz (10) and Richter (35) report that glomerulonephritis rarely healed clinically more than 1 year after onset.

1. Histologic findings and outcome of acute glomerulonephritis. Certain histologic features that might be related to the outcome of acute glomerulonephritis are presented in Tables V–IX. Some of the details of the histologic features used in

TABLE VIII

*Certain histologic findings and clinical outcome in patients with acute glomerulonephritis, grouped according to predominant lesions**

Patient	No. of glom.	Endothelial prolif.†	Leukocytes per glom.	No. of glom. with damage‡	Interstitial reaction§	Clinical outcome
		Presumed focal glomerulitis				
Ros	14	0	0	0	0	Healed
Swi	8	0	0–1	0	0	Healed
Shi	17	±, L.S.	0	12	1+	Healed
		Interstitial reaction, presumed focal glomerulitis				
Jam	9	0	0	0	2+	Chronic
		Primarily proliferative; early (within 6 weeks of onset), including focal exudation				
Eik	10	1+, L.S.	5	0	0	Healed
Dix	25	1+, L.S.	4	4	0	Healed
Mul	9	1+, Dif.	7–24	12	2+	Healed
McC	15	1+, L.S.	11	100	3+	Healed
Ser	6	2+, Dif.	13	0	1+	Healed
Vin	5	2+, Dif.	5–22	0	1+	Healed
Mol	10	2+, Dif.	12	0	1+	Healed
Har	15	2+, L.S.	1–6	0	2+	Healed
Mar	5	2+, Dif.	14–30	20	2+	Healed
San	7	3+, Dif.	9	0	0	Healed
Byr	20	2–4+, Dif.	2	15	1+	Chronic
		Primarily proliferative; late (6 weeks to 1 year after onset)				
Swe	17	1+, L.S.	0–1	0	0	Healed
Roo	10	1+, L.S.	1–2	0	1+	Healed
Dil	22	1+, L.S.	1	5	1+	Healed
Sch	36	2+, L.S.	0–1	0	0	Healed
Gav	13	2+, L.S.	0–1	0	1+	Healed
Eva	13	2+, L.S.	0–1	0	1+	Healed
Eas	11	2+, L.S.	0–1	0	1+	Healed
Moe	19	2+, L.S.	0–1	5	2+	Healed
Lar	35	3+, L.S.	0–1	0	0	Chronic
Lat	25	3+, L.S.	0–1	0	1+	Chronic
		Primarily proliferative with glomerular damage (late group only)				
Bis	7	3+, L.S.	0–1	29	2+	Chronic
Tra	15	3+, L.S.	2	33	3+	Chronic
Win	13	3+, L.S.	2	30	2+	Chronic
Gar	13	3+, L.S.	3–5	85	3+	Chronic
Rob	6	3+, L.S.	1	100	3+	Chronic
		Primarily exudative; early (within 6 weeks of onset)				
Cal	16	0	70	100	4+	Died
Ape	15	1+, L.S.	15–31	33	4+	Chronic
Lee	16	1+, L.S.	3–9	56	2+	Healed
		Primarily exudative; late (6 weeks to 1 year after onset)				
Vla	10	1+, L.S.	1–2	70	3+	Chronic
Dra	19	1+, L.S.	0–1	90	3+	Chronic
Col	12	2+, L.S.	2–3	67	2+	Healed (late)

* Only first biopsy included when more than one was obtained in a given patient. Within each group patients are arranged in relation to degree of endothelial cell proliferation.

† Endothelial cell proliferation on 0–4+ scale; location of endothelial proliferation: L.S. = lobular stalk. Dif. = diffuse.

‡ Glomerular damage = lobular necrosis or scar, capillary loop thrombus, crescent, adhesion or hyalinization.

§ 0 = no fibrosis, edema or inflammatory cells; 1+ = occasional tiny periglomerular foci; 2+ = larger cortical areas involved; 3+ = 50% or more of cortex involved; 4+ = diffuse edema or fibrosis. The interstitial reaction in all patients in the "early" group was recent except in McC who had both recent and healed fibrosis. In the "late" group the interstitial fibrosis was healed except in Col, Tra, Vla and Win in whom mixtures of recent and healed interstitial reactions were noted.

this analysis are given in Table VIII. Within each classification in this table, the patients are listed in order of increasing degrees of endothelial cell proliferation, and within each degree-of-proliferation group they are listed in order of increasing degrees of "glomerular damage." Glomerular hypercellularity and endothelial cell proliferation were graded on a scale of 0 to 4 +. When glomerular leukocytes were distributed diffusely, a single figure was given to indicate the mean number of leukocytes per glomerulus. When the exudative process was focally distributed, the range of leukocytes per glomerulus was given. The percentage of total glomeruli in the biopsy that exhibited thrombi or scars, adhesions, crescents or hyalinization was given to indicate the extent of "glomerular damage." Interstitial reaction was graded on a scale of 0 to 4 +.

The histological findings in Table VIII can be analyzed singly or together. We believe that the most useful analysis of the relation of histologic changes to the outcome of acute glomerulonephritis is based on the predominant type of lesion. Accordingly, the outcome in our 36 patients has been analyzed with respect to the severity of glomerular endothelial proliferation, exudation and "damage," and of the interstitial reaction (Tables VI, VII, IX).

TABLE IX

Relation of severity of certain histologic lesions to outcome of acute glomerulonephritis

Lesion	Severity	Clinical outcome*	
		Healed	Chronic
Endothelial cell proliferation†	0–2+	22	3
	3–4+	1	8
Glomerular damage‡	0–25%	20	4
	>25%	3	7
Interstitial reaction†	0–2+	21	4
	3–4+	2	7
Average of above§	0–2+	20	3
	3–4+	3	8

* Patients Jam, who had an unusual lesion (interstitial reaction, presumed focal glomerulitis) and who probably developed essential hypertension, and Cal, who died 32 days after onset, are excluded from this analysis.

† Severity recorded as designated in Table VIII.

‡ Per cent of glomeruli involved.

§ For purposes of calculating this average the severity of glomerular damage was assigned as follows: no glomerular damage, 0; 1–12% glomeruli involved, 1+; 13–25% involved, 2+; 26–49% involved, 3+; >49% involved, 4+.

The three patients classified as *presumed focal glomerulitis* all had rather mild clinical acute glomerulonephritis, had little or no evidence of endothelial cell proliferation, and healed, although proteinuria persisted for 9.5 months in Swi. Healing in this group was not unexpected, judging from the paucity of pathologic change observed in their biopsies. Glomerulonephritis in Swi and Ros was a "laboratory diagnosis" made on the basis of urinalyses done because of pharyngitis. Perhaps the renal lesions in these patients were similar to those in the majority of patients in epidemics such as that described by Stetson and co-workers (28). However, histologic studies in a large group of patients with mild, clinical, proven poststreptococcal glomerulonephritis, both epidemic and sporadic, will be required before much further knowledge will be gained about this type of renal response.

Patient Jam, who had interstitial reaction and presumed focal glomerulitis, apparently failed to heal, although continued evidences of renal disease could have been due to renal vascular disease and nephrosclerosis, rather than to glomerulonephritis. He was excluded from further consideration in this analysis.

Fourteen of the remaining 32 patients were biopsied within 6 weeks of onset. Eleven of the 14 (Tables V, VI) were classified as *primarily proliferative, with or without glomerular damage,* and only one of these failed to heal. The initial biopsy on this patient (Byr) showed some markedly hypercellular glomeruli (Figure 5) while others were only moderately hypercellular. The only other patient with marked proliferative glomerulonephritis who was studied early healed 4 months after onset (San, Figure 12). Mild or moderate degrees of hypercellularity were exhibited by the other 9 patients, all of whom healed regardless of the amount of glomerular damage (Table VIII).

Fifteen of the patients (Tables VI, VIII), first biopsied more than 6 weeks after onset, were classified as examples of *proliferative glomerulonephritis, with or without glomerular damage;* 7 of these failed to heal. All 7 had evidence of marked (3 to 4 +) endothelial proliferation (Figures 13, 32, 33, 35, 55, 63). The disease healed clinically in the remaining 8 patients who had

mild (1+) or moderate (2+) endothelial proliferation. The same 15 patients were reclassified in relation to the degree of observed glomerular damage rather than the degree of proliferation (Table VI). Clinical healing was achieved in 7 of the 9 patients who had little or no evidence of glomerular damage or interstitial reaction (Figures 30, 33, 38). The disease healed in only 1 (Moe) of the 6 patients who had glomerular damage in more than 20 per cent of their glomeruli. The histologic picture in the patients with chronic disease in the primarily proliferative group is one of marked lobular stalk hypercellularity and varying amounts of glomerular scarring. Bell, who first described this lesion, named it *chronic latent glomerulonephritis* (65).

Six patients were classified as *primarily exudative* (Table VIII). Three patients (Cal, Ape, Lee) were biopsied within 6 weeks of onset. Cal (Case 7), who had no evidence of endothelial cell proliferation, but did have very extensive glomerular damage and interstitial reaction, died 32 days after onset. Autopsy confirmed the biopsy findings. Ape (Case 6), who had marked but less severe glomerular damage, also had some slight endothelial proliferation. She slowly improved clinically but still had evidence of chronic glomerulonephritis 25 months after onset. Lee's first biopsy exhibited mild diffuse exudation, crescents and necrotic glomerular lobules in about one-third of the glomeruli, along with evidence of slight endothelial proliferation. His proteinuria disappeared 2 months after onset.

The remaining 3 patients (Vla, Col, Dra), who were first biopsied more than 6 weeks after onset, have been classified in Table VIII as late examples of *primarily exudative glomerulonephritis.* They all had extensive glomerular damage, interstitial fibrosis and evidence of mild (1+) or, in one case, moderate (2+) endothelial proliferation. The histologic pattern noted in these 3 patients was similar to that observed in the later biopsies on Ape. Placement of these 3 patients in the exudative group is arbitrary, since there is no objective criterion at this late date, aside from the observed extensive glomerular and interstitial scarring, to prove the initial disease was exudative in character. Two of the 3 patients have persistent proteinuria, while the clinical disease in one (Col) healed late (26 months after onset). The histologic pattern of the unscarred glomeruli in these patients is quite different from the 8 examples of chronic disease discussed earlier in Table VI.

TABLE X

Relation between over-all severity of renal lesion and several clinical features of acute glomerulonephritis *

Severity of renal lesion	Patient	Max. BUN	Max. diastolic blood pressure	Edema
		mg %	*mm Hg*	
0–1+	Swi	12	60	0
	Dil	14	84	0
	Ros	15	70	0
	Shi	17	80	0
	Swe	20	90	0
	Jam	25	90	0
	Roo	27	90	3+
2+	Sch	15	100	0
	Gav	18	80	1+
	Eas	20	100	0
	Dix	24	95	0
	Mul	24	100	2+
	Moe	25	120	0
	Har	25	108	3+
	Eva	33	90	0
	Eik	35	110	4+
3+	Dra	15†	85	2+
	Byr	17	80	0
	Ser	22	116	3+
	Lar	22†	120	1+
	Mol	24	60	0
	Bis	24†	110	2+
	Vin	25	116	2+
	Col	28†	118	3+
	McC	45	110	2+
	Lat	45	98	2+
	Lee	66	86	3+
	Mar	128	95	2+
4+	Rob	24†	120	3+
	Win	39	110	4+
	Gav	40	120	1+
	San	42	110	1+
	Ape	79	130	4+
	Vla	95	100	3+
	Col	179	116	4+
	Tra	200	98	0

* In this analysis the over-all severity of the renal lesion on a scale of 0 to 4+ is estimated from the degree of obstruction of the glomerular capillaries by endothelial cell proliferation and leukocytes, the amount of glomerular damage and the severity of the interstitial reaction.

† First blood urea nitrogen obtained more than 2 weeks after onset.

The over-all relationship of the histologic findings to the outcome of acute glomerulonephritis in 34 patients [1] is summarized in Table IX. It is ap-

[1] Patients Jam, who had an unusual lesion (interstitial reaction, presumed focal glomerulitis) and who probably

TABLE XI

Relation between several clinical features and clinical outcome of acute glomerulonephritis

	Clinical outcome	
	Healed	Chronic
Blood urea nitrogen (mg%)		
–19	6	2
20–29	11	4
30–49	4	3
50–	2	4
Diastolic blood pressure (mm Hg)		
–94	10	3
95–	13	10
Edema		
Absent	11	3
Present	12	10

parent that a relationship exists between the degree of endothelial proliferation and the incidence of chronicity. Eight of 9 patients with 3 to 4 + hypercellularity still have persistent proteinuria, while only 3 of 22 with mild and moderate degrees of endothelial proliferation have signs of chronic disease. The three exceptions (Ape, Dra, Vla, in the mild proliferative group and whose diseases failed to heal) all had had a primarily exudative reaction and considerable glomerular damage. The clinical disease healed in the three patients without diffuse endothelial proliferation. The relationship between the severity of "glomerular damage" and interstitial reaction and chronicity also was reasonably good, but more exceptions were noted.

Five patients (Col, Har, Mar, Tra, Vla) were treated with steroid hormones. At the time of treatment Tra was critically ill with severe uremic symptoms, while Vla and Mar had marked nitrogen retention and other clinical evidence of severe renal disease. Shortly after institution of steroid therapy all five patients began to show evidence of improvement. Although this improvement was rather dramatic in Tra, Vla and Mar, we are not yet prepared to state that steroids were responsible, since improvement often occurs spontaneously in severe acute glomerulonephritis.

developed essential hypertension, and Cal, who died 32 days after onset, are excluded from this analysis.

2. Histologic and clinical findings. An attempt was made to estimate from histologic evidence the reduction to be expected in glomerular filtration rate. The histologic estimations, graded on a scale of 0 to 4 +, are summarized in Table X in relation to the incidence of nitrogen retention, diastolic blood pressure and edema. In general, a reasonably good relationship between the estimated severity of glomerular lesions and the blood urea nitrogen (BUN) level was observed, although there were some impressive exceptions, several of which had reasonable explanations. For instance, in the 0 to 1 + glomerular damage group, Patients Jam and Roo, whose BUN levels were slightly increased, had relatively marked interstitial fibrosis. In addition, some glomeruli in Roo's biopsy were completely hyalinized, presumably the result of vascular nephrosclerosis. In the 3 and 4 + groups, the BUN recorded for Dra, Lar and Rob was not obtained during the first 2 weeks of their disease. Diastolic hypertension was more common among the patients with the severe glomerular damage, but again notable exceptions were apparent, as was the case with edema.

Byr and Mol represent important exceptions. Their clinical symptoms were mild, hypertension and edema did not develop, and renal functional impairment was minimal. Nevertheless, both had significant glomerular hypercellularity. The disease failed to heal in Byr.

3. Clinical findings and outcome of acute glomerulonephritis. The relationship of blood urea nitrogen levels, diastolic hypertension and edema to the outcome of acute glomerulonephritis is shown in Table XI. Again, in general, the more severe the clinical findings the less likely was acute glomerulonephritis to be followed by healing. However, exceptions were numerous and obvious.

DISCUSSION

The patients reported in this paper had sporadic acute glomerulonephritis following an infection proved to be due to a Group A hemolytic streptococcus organism. Distinctive renal lesions were observed in 32 of the 36 patients. No distinctive histologic lesions were noted in 4 patients whose diagnosis rested entirely on laboratory findings.

The fate of the pathologic changes in many of the patients in this series is still unknown. Such information will not be available until they have been followed for a longer time. Thus, some of our present conclusions about the natural history of acute post-streptococcal glomerulonephritis are subject to the restrictions of limited follow-up.

The pathology of fatal acute glomerulonephritis has been thoroughly documented. The present report is unique only in that it is a study of nonfatal cases, and that our cases have been restricted on etiologic grounds to those associated with proved streptococcal infection. The recent reports of Hutt, Pinniger and de Wardener (20), Howe (15), and Brun and co-workers (67) on smaller series of nonfatal cases certainly included some cases of post-streptococcal glomerulonephritis. However, interpretation of their reports in relation to our findings is difficult because of the lack of etiologic discrimination.

Langhans (1), Welch (41), Reichel (42), Löhlein (2) and a number of other early authors all demonstrated that the basic lesion in most fatal cases of acute glomerulonephritis was an increase in the number of cells within the glomerular capillary loops. Our observations are generally similar. It is noteworthy that these early workers observed most of the significant changes now considered to be characteristic of this disease. The main differences between their cases and ours are those of degree.

Clinical glomerulonephritis varies considerably in severity. The range of histologic damage in our series also has varied from no observable disease by light microscopy to marked acute proliferative or exudative glomerulonephritis. An endothelial proliferative response is most common; 30 of our 36 nonfatal cases showed this response, as did 57 of Bell's group of 60 fatal cases (43). No doubt most cases of acute diffuse proliferative glomerulonephritis reported in the literature are post-streptococcal in etiology. This association has been documented in most recent studies (8, 26–28, 44, 45), and has, in fact, been loosely documented in the older literature, particularly in those papers reporting classic intracapillary glomerulonephritis following scarlet fever [Langhans, 1885 (1), Löhlein, 1907 (2), and Volhard and Fahr, 1914 (3)]. Reichel in 1905 (42), even entitled his classic paper on acute glomerulonephritis, "Über Nephritis bei Scharlach." [2]

Nature of the glomerular changes. The term *proliferative* in relation to the increase in mononuclear cells within the basement membranes of the glomerular capillary loops has been questioned by MacCallum (46), Jones (47) and Grishman and Churg (48). MacCallum theorized that no real reason existed for assuming that inflammation in the glomerulus should differ from inflammation elsewhere in the body. He decided, on morphologic grounds, that the hypercellularity of acute glomerulonephritis was due to mononuclear cell infiltration into the glomerular capillary loops. We noted mitosis in cells inside and attached to the glomerular capillary basement membrane (by definition, endothelial cells) during the early phase of the clinical disease in 2 patients. This is strong evidence against MacCallum's theory. We are not alone in this observation, since mitoses have been noted previously by a number of workers (5–7, 49, 50). Furthermore, mitoses are commonly observed in capillary endothelial cells in the initial phases of "Masugi" antikidney serum nephritis in the rat (51).

Farquhar, Vernier and Good's (17) electron micrographs of hypercellular glomeruli from the initial phases of acute proliferative glomerulonephritis and our micrographs (Figures 27, 28) of diseased glomeruli from the subsiding phase show cells with characteristic cytoplasmic inclusions of material with the same morphologic qualities and electron density as glomerular capillary basement membrane. Such inclusions have never been reported in mononuclear inflammatory cells. It seems clear from these observations and arguments that good evidence exists to indicate that *proliferative* is a valid term descriptive of the phenomena occurring in this disease.

Two patients in our series showed marked glomerular hypercellularity, which was primarily due to an increase in the number of polymorphonuclear neutrophiles within the glomeruli. Minimal endothelial cell proliferation was present in 1 patient, but was overshadowed by the exudative response. Glomerular necrosis, crescents, and other signs of severe renal disease were common in these pa-

[2] See Reichel's paper for a list of 12 earlier papers on *Scharlachnephritis.*

tients but also occurred in patients with the primarily proliferative disease. Severe acute exudative glomerulonephritis, as observed in Cal, is very similar to the extracapillary glomerulonephritis described first by Langhans in 1885 (1). We suspect that acute exudative glomerulonephritis is the antecedent lesion of so-called subacute glomerulonephritis.

Bell described three cases of acute exudative glomerulonephritis (4, 43). He stated that they were associated with staphylococcal infections, the symptoms of which overshadowed the nephritis. However, within our series, cultures of blood from the biopsy needle in all of our patients were sterile, as were cultures of urine. We have no explanation as to why the kidneys of these patients responded with so much more exudation than proliferation.

That a mixed proliferative and exudative response should occur in post-streptococcal acute glomerulonephritis is not too surprising, but the factors and mechanisms involved are unknown. At present we postulate that most of the lobular scars noted in both the proliferative and exudative groups are monuments of previous focal areas of glomerular exudation or glomerular capillary thrombosis. However, we have little direct evidence to submit on this point because of the relatively few patients with marked glomerular exudation in whom we obtained sufficient serial biopsies. Nevertheless, 8 patients (Table V, group labeled proliferative plus glomerular damage) showed evidence of glomerular proliferation (lobular stalk hypercellularity) along with lobular scars and healed crescents, which could be examples of a mixed response. The disease healed in only three of these patients.

Pathogenesis of chronic glomerulonephritis. Textbooks have taught for many years that acute glomerulonephritis may fail to heal in some patients (4, 12, 34). After clinical subsidence of the acute attack, the only objective sign of disease may be persistent proteinuria, although microscopic hematuria and cylinduria are not infrequent. This phase, often labeled *latent* or *transitional* chronic glomerulonephritis may persist for 10 to 30 years before renal insufficiency, azotemia and finally death in uremia develop (4, 9–11).

The pathogenesis of chronic glomerulonephritis, as defined above, recently has become a subject of controversy. Some workers, Rammelkamp in particular (27), have questioned whether chronic glomerulonephritis is ever the result of an antecedent attack of post-streptococcal acute glomerulonephritis. This viewpoint appears to be based on observations of epidemics of post-streptococcal (Type 12) acute glomerulonephritis in military recruits and school populations (28, 45). As evidenced by disappearance of proteinuria and microscopic hematuria, the renal lesion in practically all patients observed under epidemic conditions apparently healed. However, the majority of the diagnoses of acute glomerulonephritis were made on the basis of mild proteinuria and microscopic hematuria appearing during convalescence from Type 12 streptococcal pharyngitis. Very few patients had edema, hypertension or renal functional impairment. We suspect that the majority of such patients would show few or no lesions on renal biopsy (similar to our presumed focal glomerulitis group, Patients Swi, Ros and Shi).

Information obtained from studies of epidemic nephritis is of obvious importance and value but is not necessarily applicable to acute glomerulonephritis as it occurs sporadically in the adult population. Twelve (or possibly 13 if Jam is included) of our 36 patients with sporadic acute glomerulonephritis following proved streptococcal infections have developed clinical evidence of chronic glomerulonephritis; evidence was obtained that the streptococcus involved in 3 of these 12 patients was Type 12. No evidence of prior renal disease was obtained in any of our patients, and latent periods between the onset of infection and acute nephritis in excess of 1 week were the rule. The "latent period" in exacerbations in chronic glomerulonephritis is generally less than 5 days (26). Other authors have reported similar incidences of chronicity following acute glomerulonephritis (10, 11, 35, 44, 52–54), in some instances associated with proved hemolytic streptococcal infections.

Even though prognosis for healing is better in children (8, 44, 49, 52, 55–58) some authors (59) have reported instances of acute glomerulonephritis following streptococcal infections that failed to heal [also Case 4 of Earle, Taggart and Shannon (60), and Case VN of Wertheim and

co-workers (61)]. Other cases in children of acute glomerulonephritis that failed to heal, in which proof of initial association with a streptococcal infection was lacking, have been reported by several authors (8, 12). The reason for the more favorable prognosis in children is not known. Perhaps examination of the urine following an infection is performed more often in children than in adults, and hence more instances of mild disease are discovered in the young. Pediatricians have often commented (52) that even children very ill with acute glomerulonephritis often recover completely. Perhaps children develop a more severe clinical response to lesser degrees of glomerular exudation or proliferation than do adults. Our data for adults with sporadic poststreptococcal acute glomerulonephritis suggest that a direct relationship exists between the severity of the clinical attack (hematuria excluded) and the prognosis for development of chronic disease (23). Mild clinical attacks usually have healed. The incidence of healing after acute glomerulonephritis, however, is obviously influenced by criteria of diagnosis and for healing (57), as well as by age, epidemicity and other unknown factors.

Classic clinical subacute glomerulonephritis (extracapillary glomerulonephritis of the earlier authors) was not observed in our series, although Patient Cal had typical changes which, however, developed acutely rather than subacutely. Two similar cases, kept alive with the artificial kidney for 75 days, have recently been described (63). The use of the term *subacute glomerulonephritis* to describe other circumstances, such as an episode of the nephrotic syndrome occurring in the course of chronic glomerulonephritis, is confusing. Volhard's (64), Bell's (65) and Ellis's (9) descriptions of the patient who develops acute glomerulonephritis, who never clinically recovers, who dies within 3 months to 1 year of onset with hypertension and uremia, and who, at autopsy, has large, scarred kidneys with many obliterated glomeruli, crescents and severe interstitial fibrosis and edema, delineate very adequately a rather well defined, although uncommon, entity. Our data suggest that subacute glomerulonephritis may be a consequence of a severe initial exudative response.

Good and Vernier (21) have suggested that acute hemorrhagic glomerulonephritis and chronic glomerulonephritis may be independent diseases. They speculate that the occasional apparent progression of acute glomerulonephritis to the chronic form may be due to repeated exacerbations of acute disease, each exacerbation producing further cortical destruction. They cite two children who showed this type of progression clinically and upon serial renal biopsy.

It seems unlikely that repeated exacerbations are the usual cause for progression to chronic glomerulonephritis in adults. None of our 13 patients who developed chronic disease in the present study has so far had exacerbations, although we have observed exacerbations in other patients with chronic glomerulonephritis. Earlier studies on the natural history of poststreptococcal glomerulonephritis also support this conclusion. Loeb, Lyttle, Seegal and Jost (62) clearly showed that recovery is usually permanent following healing in acute glomerulonephritis, since none of 10 patients they followed through Group A streptococcal infections developed chronic glomerulonephritis. Seegal and co-workers (26) defined the clinical characteristics of exacerbations in chronic glomerulonephritis. Their measurements of renal function in such patients demonstrated that exacerbations only rarely accelerated the progression of chronic glomerulonephritis.

Ellis (9) proposed that "Those patients who develop chronic glomerulonephritis after an acute attack have had some renal damage as evidenced by persistent albuminuria, whence we presume there is also some permanent vascular damage which leads to renal ischemia; this renal ischemia eventually leads to renal hypertension, and so a vicious circle is introduced, hypertension causing vascular damage leading to further ischemia and augmented hypertension." Evidence against this point of view includes the absence of any acute vascular damage in renal biopsies we obtained during the acute or the early chronic phases of glomerulonephritis. Our observations, as well as those of Murphy and Schulz (10), that hypertension is an uncommon finding during much of the chronic latent phase of glomerulonephritis, are further points against Ellis's viewpoint. If permanent vascular damage and renal ischemia are to be interpreted as the cause of progressive renal dis-

ease, hypertension would be expected to persist from the acute into the chronic latent phase, or at least to develop early in the chronic phase. Finally, the majority of our patients, even with severe histologic evidence of early chronic glomerulonephritis and considerable interstitial fibrosis, had no evidence of vascular disease. The above remarks are not intended to deny that hypertension or renal vascular disease, if present, could accelerate the progression of chronic glomerulonephritis.

The idea that the glomerular changes induced by the acute attack may become slowly progressive best fits our data concerning the development of chronic glomerulonephritis. We postulate that those patients in the chronic latent group who have prominent foci of lobular stalk thickening and hypercellularity slowly will develop more marked lobulation, gradual hyalinization and scarring of the glomeruli. When glomerular scarring becomes sufficiently severe to compromise efferent arteriolar blood flow to the cortex, ischemic necrosis with consequent scarring and loss of tubular mass should develop. These progressive changes would lead eventually to small, contracted kidneys, azotemia, hypertension, and death in uremia.

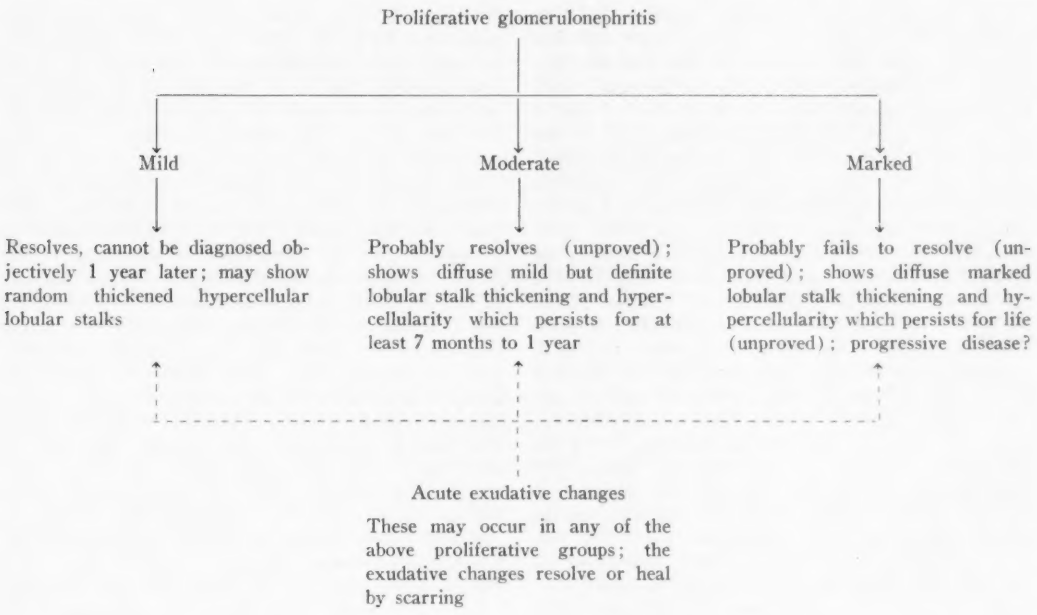
Lobular stalk thickening and hypercellularity were very prominent in 8 of our patients in the latent phase of a chronic glomerulonephritis (Figures 13, 33, 55, 63). These lesions were clearly discerned by Bell in 1936 in his classic paper on chronic glomerulonephritis (65). He named this pathologic lesion *chronic latent glomerulonephritis.* He described it in 8 patients who died of intercurrent diseases while in a clinically silent phase, except for proteinuria, of chronic glomerulonephritis. Bell also noted the similarity of the glomerular lesion of the end-stages of chronic azotemic glomerulonephritis to the lesion of the lobular stalks of the chronic latent phase. He implied that the initial glomerular lesion was progressive and led to the chronic disease.

Both Bell (43) and McGregor (5) noticed the hyaline fibers illustrated in Figure 12. They were present in foci of endothelial cell proliferation during the acute phase. Similar material, clearly shown by electron microscopy to have structural characteristics simliar to the lamina densa of the glomerular capillary basement membrane, is demonstrated in regions of lobular stalk hypercellularity in Patient Mol (Figures 26–28). This material lacks the periodicity of collagen, and, therefore, is not likely to represent scar tissue. Bell (65) implied that these hyaline fibers ultimately lead to the glomerular scarring of chronic glomerulonephritis. This is an attractive hypothesis. However, many of our patients whose disease healed clinically (e.g., Mol, Ser) initially exhibited similar fibrillar material.

Although glomerular damage such as lobular scars, adhesions and crescents usually were associated with the development of chronicity, this was not inevitably the case (e.g., Col, Lee). Apparently these lesions can heal in such a way that clinical cure is possible. Actually, glomerular scarring from the primarily exudative reaction should be a self-limited process. Healing, therefore, might occur late in those patients with a moderately severe acuté exudative glomerulonephritis and little endothelial response. A possible clinical counterpart of this phenomenon is suggested by a few patients that Addis (12) reported as having healed several years or more after onset. He aptly labeled this process "healing with a defect."

Diffuse membranous glomerulonephritis (66) was never observed in any of the 36 patients in this series. However, a few patients showed focal basement membrane thickening. For example, some of the peripheral capillary loops of Gar, who had the nephrotic syndrome at the time of biopsy, were thickened due to fibrinoid deposits. Focal thickening of capillary loops also was occasionally observed in areas of thrombosis or necrosis in either focal or diffuse exudative glomerulonephritis. Here the thickening was probably due to deposits of fibrin or other protein on either side of the basement membrane. However, the lack of much significant basement membrane disease together with the frequent finding of thickened basement membranes in idiopathic instances of the nephrotic syndrome is strong evidence that post-streptococcal chronic glomerulonephritis and membranous glomerulonephritis are not only histologically but also etiologically distinct.

The course of the histologic changes in post-streptococcal diffuse proliferative glomerulonephritis is summarized in the following scheme.


Proliferative glomerulonephritis
Mild
Moderate
Marked
Resolves, cannot be diagnosed objectively 1 year later; may show random thickened hypercellular lobular stalks
Probably resolves (unproved); shows diffuse mild but definite lobular stalk thickening and hypercellularity which persists for at least 7 months to 1 year
Probably fails to resolve (unproved); shows diffuse marked lobular stalk thickening and hypercellularity which persists for life (unproved); progressive disease?
Acute exudative changes
These may occur in any of the above proliferative groups; the exudative changes resolve or heal by scarring

SUMMARY

The histopathologic findings and clinical course of 36 patients with post-streptococcal acute glomerulonephritis are reported. All patients were first biopsied within 6 months of onset and have been followed until they healed clinically or developed clinical chronic glomerulonephritis; 56 percutaneous biopsies were obtained.

The 36 patients exhibited much of the spectrum of the renal response to a nephritogenic streptococcal infection. Included are 7 patients whose disease was so mild that the diagnosis rested primarily on laboratory findings and 29 who had overt clinical disease of slight to marked severity. One patient died while anuric 32 days after onset.

Distinctive renal lesions were observed in the glomeruli of 32 patients; 26 showed mild, moderate or marked diffuse proliferative glomerulonephritis, while 6 showed diffuse exudative glomerulonephritis. The glomeruli of the remaining 4 patients showed no distinctive lesions: They have been classified as presumed focal glomerulonephritis; 1 of the 4 had a subacute interstitial inflammatory reaction.

The nephritis had not healed at the last follow-up in 13 patients. In general, these patients had more lobular stalk hypercellularity and evidences of glomerular damage than did those patients who achieved clinical healing.

The relationship of the glomerular lesions observed to the severity of the clinical disease and to the pathogenesis of chronic glomerulonephritis is discussed.

CASE HISTORIES

Case 1. An 18 year old white male (Eva) noted a sore throat and fever which disappeared after several days without treatment of any sort; 10 days later he suddenly developed malaise, anorexia, nausea and pain in one foot. The next day he was admitted to the hospital with pain in both ankles, both knees and both shoulders. His past history was noncontributory except for rheumatic fever 3 years previously. He was treated at that time with steroids for 4 weeks, and was told there was no cardiac involvement.

On admission his temperature was 101.6° F, the blood pressure 130/90 mm Hg, and the joints noted above were tender. Except for a grade 1 systolic murmer at the apex, the remainder of the physical examination was normal. Laboratory tests revealed no anemia, but a moderate leukocytosis and an erythrocyte sedimentation rate (ESR) of 32 minutes in 1 hour (Westergen) were present. The C-reactive protein test (CRP) was positive. On admission, 11 days after onset of the sore throat, the urine had a specific gravity of 1.018, contained no protein, and the centrifuged sediment revealed

only a few leukocytes. A throat culture revealed no hemolytic streptococci, but the serum ASO titer was more than 1,000 Todd U. Over the next 7 months this gradually decreased to 200 U. Serum antibodies were demonstrated against Type 12 streptococci 5 and 6 months after onset, but not against Types 6, 30 or Red Lake. Serum complement was not measured until 5 months after his sore throat. Monthly tests thereafter were all normal. He was placed on bed rest and aspirin, with prompt subsidence of fever and symptoms. Four days after admission, or 15 days after the onset of the sore throat, a routine examination of the urine revealed 2+ protein, numerous erythrocytes and occasional granular casts. At no time was edema, hypertension or gross hematuria noted. The day after admission the BUN was 33 mg per 100 ml; 6 days later this had decreased to 19.5 mg per 100 ml and subsequently was normal. Urine protein increased to 4+. Two months after discovery of abnormal urine, he was excreting 1.8 g per day, and the urinary sediment was still loaded with erythrocytes. At this time the PSP test revealed 50 per cent excretion in 45 minutes, and the urine specific gravity was 1.020 in a concentration test. The proteinuria then gradually improved and disappeared by Day 84 after onset. Some microscopic hematuria persisted until the end of observation, 7 months after onset.

A renal biopsy, obtained 144 days after onset of the renal disease, and 61 days after disappearance of proteinuria, revealed 13 glomeruli, all of which exhibited a moderate degree of lobular stalk hypercellularity (Figure 34). No other glomerular lesions, tubular damage or arteriolar disease was noted. Attempts to discontinue salicylate therapy resulted in recurrences of fever and joint pains until 6 months after onset, when therapy was successfully terminated. He was discharged 7 months after onset, asymptomatic and with normal physical and laboratory findings, except for some erythrocytes in the centrifuged sediment. He has been lost to follow-up.

Case 2. An 18 year old white male (Mol) was treated with aspirin for tonsillitis, with subsidence of symptoms within a few days; 8 days later the urine was examined and found to be normal. However, 22 days after onset of tonsillitis, another urinalysis revealed proteinuria. The next day the tonsillitis recurred. One day later the urine contained 4+ protein, erythrocytes and leukocytes too numerous to count and occasional granular casts; a urine culture was sterile. He was placed on penicillin therapy for 2 weeks. The ASO titer was 625 U, but gradually decreased to 100. At 2 and 6 months after onset serum antibodies against Types 6, 12, 30 or Red Lake streptococci were not present. Serum complement was 30 U (normal: 35 to 50 U) near the onset and ranged between 26 and 34 U during the first 10 weeks after onset. Neither hypertension nor edema developed. The urinary specific gravity in casual specimens ranged between 1.020 and 1.035 throughout the period of observation. PSP excretion in 15 minutes was 35 per cent on admission and 30 per cent 2 months later. The BUN was 24 mg per 100 ml on admission and the urea clearance 48 ml per minute. The BUN decreased to 13 mg per 100 ml within 1 month. The urine became protein-free 2.5 months after onset, while a week earlier an Addis count revealed normal numbers of red blood cells, leukocytes and casts. However, 3.5 months after onset, microscopic hematuria recurred for several days, and one specimen contained 1+ protein. Numerous subsequent urines, however, were normal.

The first renal biopsy (Figure 9) was done 22 days after onset. Proteinuria and marked microscopic hematuria were still present. The BUN had just returned to normal. All the glomeruli were hypercellular, due in part to moderate endothelial cell proliferation, and in part to the presence of as many as 10 eosinophilic and 2 neutrophilic polymorphonuclear leukocytes in the capillary loops of each glomerulus. The capillary loops were decreased in caliber. Many of the proximal tubules contained erythrocytes, while many of the distal tubules contained cellular (polymorphonuclear leukocyte) casts. The cortical architecture was partially disrupted by small foci of immature fibrosis tissue. The arteries and arterioles were normal. A second biopsy was performed 114 days after onset. The urine had been normal for almost 1 month before this biopsy was obtained, but this biopsy was secured during an episode of 1+ proteinuria and microscopic hematuria (44 million erythrocytes in 12 hours) which lasted for approximately 1 week. The biopsy (Figures 23 and 24) showed hypercellular glomeruli. Only an occasional polymorphonuclear leukocyte was seen in the capillary loops. The hypercellularity was focal in nature and confined to the lobular stalk area. The capillary loops were more patent than on the first biopsy. However, adhesions between capillary loops and Bowman's capsule were noted in 3 of the 34 glomeruli present in this biopsy. Occasional small foci of interstitial fibrosis were present.

The urine subsequently examined frequently remained normal. A third biopsy, obtained 205 days after onset and 136 days after disappearance of proteinuria contained 9 glomeruli, 1 of which was hyalinized. Some lobular stalk hypercellularity persisted (Figures 25–28), as did a few foci of mature interstitial fibrosis.

Case 3. A 40 year old white male (Ser) developed gross hematuria 13 days after onset of a sore throat, bronchitis and a temperature of 100° F. Two years previously his blood pressure and urine were normal. Eight days before the gross hematuria he had noted puffy eyes. He visited his physician, who found his blood pressure to be 172/116, with rales at both bases, and with considerable edema of the face and legs. Unfortunately, the urine was not examined, but he was treated with digitoxin and tetracycline hydrochloride. The blood pressure had decreased to 130/94 mm Hg and the edema had disappeared when the gross hematuria developed. The urine remained dark and he was admitted to the hospital 25 days after the beginning of the edema and 17 days after the gross hematuria. On admission the physical examination was normal except for obesity and a blood pressure of 150/96. The ASO titer was 500 U, but after 1 month began to decrease, being 166 U 5 months after onset. On four occasions

between 3 and 21 months after onset, serum antibodies against Type 6, 12, 30 or Red Lake streptococci could not be demonstrated. The serum complement was 20 U on admission, but returned to normal within 1 month. The urine contained 4+ protein, many red blood cells and casts (including blood cell casts). The BUN was 17 mg per 100 ml. The urea clearance was only 18 ml per minute shortly after admission, but within 2 months had increased to 38 ml per minute. PSP excretion was 24 per cent in 15 minutes, and a concentration test revealed a urine specific gravity of 1.020. The hypertension disappeared after 3 days in the hospital. Almost daily urines contained protein during the first 2 months of hospitalization, but thereafter were protein-free. Microscopic hematuria, however, persisted for another 4 months before disappearing.

Four renal biopsies were performed, the first 30 days after onset of edema at a time when proteinuria and hematuria were considerable and the urea clearance was 18 ml per minute. All lobules of all glomeruli were diseased, showing proliferative glomerulonephritis of moderate severity (Figures 3, 4). Numerous polymorphonuclear leukocytes, including eosinophiles, were present in the capillary loops. As many as 15 leukocytes were counted in a single 2 μ section of a glomerulus. Many of the capillary loops were patent but others were obscured by the increased endothelial cells. The tubules contained desquamated brush border material but were otherwise normal and contained no casts. The arterioles were normal and no interstitial reaction was present. The second biopsy (Figures 14–16) was obtained 80 days after onset, 1 week before the proteinuria disappeared. At this time the Addis count revealed 48 million erythrocytes in 12 hours. The urea clearance had increased to 38 ml per minute. Again, all glomeruli were hypercellular, but the hypercellularity was now prominent in the lobular stalks of the glomeruli. As many as 6 polymorphonuclear leukocytes were present in each glomerulus. Several very tiny foci of interstitial fibrosis, not observed in the first biopsy, were present in the cortex, but the cortical architecture was generally intact (Figure 14). The cytoplasm of most of the proximal convoluted tubules contained hyaline droplets. The arteries and arterioles were normal. The third biopsy (Figure 17) was obtained 5 months after onset and more than 2 months after the disappearance of proteinuria. The urea clearance was now 43 ml per minute and a 12 hour urine specimen contained 6 million red blood cells. The histologic findings in this biopsy were very similar to those of the second biopsy, but no glomerular leukocytes or interstitial reaction were present.

After this biopsy the urine remained normal; 26 months after onset a fourth biopsy contained 2 glomeruli, 1 of which was normal while the other revealed only slight lobular stalk hypercellularity (Figure 18).

Case 4. A 24 year old Puerto Rican male (San) noted red urine approximately 6 weeks after a mild sore throat. As far as he knew, he had never had renal disease before. The red urine persisted for 2 or 3 days. Approximately 2 weeks later he complained of cough and right lower chest pain. A physician found a temperature of 103° F and diagnosed pneumonia; the patient was given an injection and some pills. Eight days later he noted puffy eyes, and 2 days after this he was admitted to the hospital. He gave a history of passing worms while living in Puerto Rico and frequent loose stools since the age of 15. On examination he appeared acutely ill. His face and eyes were puffy; his temperature was 101° F, the pulse 74 and the blood pressure 180/102. A generalized lymphadenopathy was noted, but the pharynx appeared normal. A soft blowing systolic murmur was heard at the upper left sternal border. The liver was felt two finger-breadths below the costal margin and was tender. The ASO titer was 250 U on admission, increased to 500 and then gradually decreased to 50 U. Five sera obtained 2 to 24 months after onset contained no antibodies against Type 6, 30, 12 or Red Lake streptococci. The serum complement was very low at first (less than 7 U), but after 1 month began to increase, becoming normal after 5.5 months. Complicating diseases included Manson's schistosomiasis and an active duodenal ulcer. The venous pressure on admission was 220 mm saline. A chest roentgenogram revealed a slightly enlarged heart and pulmonary congestion. On bed rest these findings cleared within 2 weeks, and were associated with a 12-pound weight loss. The urine on admission contained 4+ protein, and by Addis count a 12 hour specimen had 8.4 million red blood cells and 204,000 casts. The proteinuria rapidly decreased so that 6 weeks after onset of gross hematuria, only a trace of protein remained. For the next month, half the urines were protein-free, and thereafter were consistently normal. On admission the BUN was 30 mg per 100 ml, increased within a week to 42, and then returned to normal after another 3 weeks. The PSP excretion near admission was 14 per cent in 15 minutes, but 1 month later had increased to 31 and then 37 per cent. The urea clearance, 1 month after onset, was 63 per cent of normal, and thereafter varied between 75 and 109 per cent of normal. Concentration tests during the first month yielded a maximal urine specific gravity of 1.013.

The first renal biopsy was obtained 32 days after onset of gross hematuria, and at a time when edema, hypertension and a venous pressure of 150 mm saline were still present. The 7 glomeruli in the specimen were very hypercellular (Figures 1, 2, 12). The capillary loops were obscured. Each glomerular lobule was club-shaped, due to the excess number of cells. The glomeruli contained as many as 9 polymorphonuclear leukocytes, and several had 1 or 2 eosinophiles. Considerable protein precipitate was present in Bowman's space of each glomerulus. The cytoplasm of the proximal tubule cells contained hyaline droplets, but no casts were visible. The arteries and arterioles were normal. No interstitial reaction was noted. The second renal biopsy (Figures 21, 22) was performed 73 days after onset, at which time the urine contained only a trace of protein and a few erythrocytes. All 14 glomeruli in the biopsy were diseased, but the hypercellularity was

now focal in nature and confined to the lobular stalks. Some lobular stalks were much more involved than others (Figure 22). In the regions of the hypercellularity, PAS-positive, aniline blue-positive material was present. Most of the glomeruli contained 1 or 2 leukocytes, but one contained 5, including 3 eosinophiles. In contrast to the first biopsy, the glomerular capillary loops were prominent and patent. Some hyaline droplets were still present in the proximal cytoplasm of the tubular cells. The arteries and arterioles were normal, except for a tiny focus of subintimal fibrosis in a branch of a medium-sized artery. A third biopsy, taken 169 days after onset and several months after proteinuria had disappeared, unfortunately consisted mostly of medulla and had but 1 glomerulus. This glomerulus showed focal lobular stalk hypercellularity similar to that observed earlier.

Case 5. A 19 year old white male (Tra) first noted gross hematuria approximately 1 month after an upper respiratory infection. He had no evidence of prior renal disease, and 2 months earlier, blood pressure and urine on admission to the Army were normal. The gross hematuria cleared after several days. The patient did not consult a physician until 18 days later when gross hematuria and malaise recurred. He had no edema and on admission the blood pressure was 124/80. The physical examination was normal. The urine was dark red with a specific gravity of 1.007, and contained 100 mg protein per 100 ml, erythrocytes too numerous to count, many red blood cell casts, and 8 to 10 leukocytes per high-power field. The hemoglobin was 11 g per 100 ml blood and the BUN was 125 mg per 100 ml. Unfortunately, a throat culture was not obtained, but the ASO was 250 U shortly after admission, increased to 500, and then gradually decreased to 125 U. Serum obtained 2, 2.5, and 3.5 months after the first episode of hematuria did not contain antibodies against Type 6, 12, 30 or Red Lake streptococci. Serum complement, first measured 1 month after onset, was slightly decreased (31 to 37 U) but then increased above normal (68 U) and subsequently remained normal (45 U). An oliguria of 200 to 500 ml urine per day was present during the first 2 weeks in the hospital, as were nausea and frequent vomiting. The blood pressure increased to 160/98, but edema never developed. Ten days after admission the patient was critically ill with a BUN of 139 mg per 100 ml, a plasma potassium of 7.5 mEq per L, a pericardial friction rub and a grossly abnormal electrocardiogram. At this time therapy with intravenous adrenocorticotropic hormone (ACTH) was instituted because of the desperate situation. With 2 days a dramatic improvement in all features was noted. Within 2 weeks the BUN had decreased to 60 mg per 100 ml, and was normal (13 mg per 100 ml) by 7 weeks after admission. The urine also rapidly improved, and by the seventh week contained only 1 + protein and a few erythrocytes.

A renal biopsy, obtained in the seventh week (10.5 weeks after the first episode of gross hematuria) contained 15 glomeruli (Figures 63, 64). Both cortex and medulla were present. The architecture of the cortex was distorted by the presence of a rather diffuse interstitial fibrosis. In some areas of dense fibrosis the tubules were atrophic and lined by regenerative-type epithelium. Scattered inflammatory cells, chiefly lymphocytes and macrophages with golden-brown iron-positive pigment in their cytoplasm, were present in some of the areas of fibrosis. This pigment stained negatively for hemoglobin. The glomeruli were all diseased, showing foci of lobular stalk hypercellularity. Several adhesions between capillary loops and Bowman's capsule were present. One large healing crescent was observed (Figure 64); 0 to 1 polymorphonuclear leukocyte was seen in each glomerulus. No vascular disease was observed. Red blood cells, heme casts and hyaline casts were common. After the biopsy, renal function continued to improve so that 5 months after onset PSP excretion had increased to 30 per cent in 15 minutes, and on concentration the urine achieved a specific gravity of 1.030. The urine, however, continued to have 1 + protein and 2 to 3 erythrocytes per high-power field.

A second renal biopsy obtained at this time revealed 11 glomeruli, all of which exhibited thickened lobular stalks with focal hypercellularity. Two glomeruli contained healed crescents and several had adhesions between capillary loops and Bowman's capsule. The cortical architecture was disrupted by multiple small foci of interstitial fibrosis composed of mature collagenous material, some of which contained small collections of inflammatory cells, chiefly lymphocytes. Three months later, or 8 months after onset, the patient had no symptoms, but his urine still contained 1 + protein.

Case 6. A 37 year old white housewife (Ape), whose son had just recovered from tonsillitis, developed a headache, rhinorrhea and a slight sore throat. Her temperature was 102° F. Without treatment the symptoms subsided over several days. One week later, a cough productive of a small amount of sputum developed; 3 days later, or 10 days after the sore throat, she came to the hospital because of this cough. Her two pregnancies, 6 and 1 years previously, had been uneventful—without hypertension, urinary abnormality or toxemia. Two years before she had had a cholecystectomy for calculi and jaundice. Blood pressure and urine were normal at this time. On admission she appeared very obese. Edema was barely demonstrable, but she said that her feet and face felt tight and puffy. Shortly after admission diuresis occurred. She lost 20 pounds in 10 days. Her blood pressure was 170/90. This gradually increased to a maximum of 190/106 2 weeks after admission. A small hemorrhage was noted in the left eyeground. The pulmonic second sound was accentuated and louder than the aortic second sound. This was reversed after several weeks in the hospital. The remainder of the physical examination was within normal limits. A throat culture revealed Group A streptococci, but unfortunately the culture was discarded before the organism could be typed. Roentgenograms of the chest revealed pulmonary congestion and probably bronchopneumonia. She was treated with penicillin for 2 weeks.

The bronchopneumonia and pulmonary congestion gradually cleared. Subsequent throat cultures did not reveal hemolytic streptococci. The ASO titer was greater than 833 U on admission. This gradually decreased to 250 U over the next 2 years. During this time 6 sera failed to reveal antibodies against Type 6, 12, 30 or Red Lake streptococci. The first serum complement was 37 U, but thereafter most values were in the range of 50 to 70 U. On admission the urine contained 4+ protein, many erythrocytes, frequent leukocytes and casts. The BUN was 61 mg per 100 ml. One month after admission the urea clearance was 12 per cent of normal, and the PSP excretion 1 per cent in 15 minutes. The hemoglobin was 10.4 g per 100 ml blood, subsequently decreasing to 7.5. No cause for the anemia, other than azotemia, could be demonstrated. The hemoglobin increased to 13.1 g per 100 ml within 6 months without treatment.

The clinical course and times of renal biopsies in this patient are shown in Chart 2. Three years after onset the blood pressure ranged between 106/65 and 128/90, the urine still contained from 30 to 100 mg protein per 100 ml, occasional erythrocytes and hyaline and granular casts. The urea clearance varied between 79 and 88 per cent of normal. During the follow-up period she had three upper respiratory tract infections and recurrent attacks of sinusitus. None of these was associated with demonstrable hemolytic streptococci. ASO, ASK and AH titers did not increase after any of the infections, nor did exacerbations in chronic glomerulonephritis ensue.

The first renal biopsy, obtained 22 days after onset, contained 13 glomeruli (Figures 40–44). The cortical architecture was disrupted by a generalized edema and immature fibrous tissue. In addition, two small perivascular foci of chronic inflammatory cell infiltration, chiefly lymphocytes and monocytes were present. All glomeruli were diseased. Some glomerular lobulation and a slightly increased number of cells in some lobular stalks were also noted. These areas contained an increased quantity of PAS-positive, aniline blue-positive homogenous material. Numerous polymorphonuclear neutrophiles were present in the capillary loops—as many as 32 per glomerulus. Adhesions between capillary loops and Bowman's capsule and several crescents were present. The arteries and arterioles were normal. Numerous red blood cell and hyaline casts were present in dilated tubules. The second biopsy, obtained 69 days after onset, contained 14 glomeruli (Figures 48–50). The cortical architecture was distorted by foci of mature scar tissue which contained numerous atrophic and regenerating tubules. Edematous connective tissue was present in some areas. All the glomeruli were diseased. They showed lobulation with some slight hypercellularity (Figure 49). In contrast to the first biopsy, almost no inflammatory cells were noted in the glomerular capillary loops. Some healing crescents and adhesions also were present (Figure 50). Hyaline droplets were noted in a few of the epithelial cells over some capillary loops in a few glomeruli. Many red blood

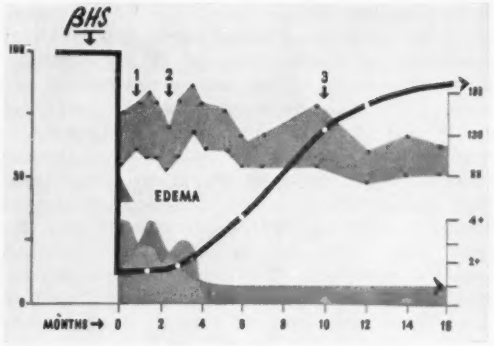


CHART 2. CLINICAL COURSE AND BIOPSIES OF CASE 6. Renal function as per cent of normal is on the left, and the black line represents a plot of estimated renal function at various times after onset. Blood pressure is in mm Hg, proteinuria is on a 0 to 4+ scale, and hematuria is shown by the gray areas on the proteinuria scale. Biopsies were taken at 1, 2 and 3.

cells, heme and hyaline casts were seen in tubules. A few red blood cells were present in Bowman's space of a few glomeruli. One arteriole had a focus of medial and intimal hyalinization. A third biopsy, obtained 307 days after onset, was adequate in size (7 × 1 mm) but was mostly medulla. The medulla was normal except for occasional hyaline casts in the thin loops of Henle. The 1 mm of juxtamedullary cortex present contained an area of scarring with a dense focal lymphocytic infiltration. A single glomerulus was lobulated and scarred. A small tag of nonfibrotic cortex was present.

Case 7. A 34 year old Negro (Cal) was given a single injection of 300,000 U of procaine penicillin by his physician for a moderately severe sore throat. Four days later he noted dark brown urine, which persisted for several days. One week after this he noted decreased urine output and progressive swelling of the feet, abdomen and eyes. During the following week he gained 50 pounds, and finally came to the hospital 3 weeks after the beginning of the sore throat. He knew of no previous renal disease. Physical examination revealed a huge Negro who weighed 355 pounds and was 6 feet, 2 inches tall. His blood pressure was 180/116. Puffy eyes and 2+ pretibial edema were present; the fundi were normal. A grade 3 systolic murmur was heard over the precordium. A throat culture revealed no hemolytic streptococci, but the ASO titer on admission was 333 U and increased to 400 U 10 days later. Serum type-specific antibodies could not be studied, since the patient was on antibiotics throughout his hospital stay. Despite a leukocytosis of 23,000 per mm^3, the serum complement was significantly decreased to 23 U.

The urine contained 4+ protein, many erythrocytes, 10 to 20 leukocytes per high-power field, and frequent granular and fatty casts. The BUN was 134 mg per 100 ml on admission and increased to 177, while the

plasma potassium increased from 6.7 to 8.2 mEq per L before the first of three dialyses on the artificial kidney was begun. A roentgenogram of the chest revealed general enlargement of the heart and pulmonary congestion. The daily urine output varied between 115 and 180 ml. Each of three hemodialyses resulted in transient improvement in clinical and blood chemical findings. Despite the large size of the patient, renal biopsy was performed 30 days after the onset of gross hematuria (Figures 45–47). It was hoped that the biopsy would reveal whether or not further dialyses should be performed. The biopsy contained 16 glomeruli; the cortical architecture was disrupted by diffuse recent fibrosis and edema. The proximal tubules were widely separated by this material and few normal proximal tubules were present; the proximal tubules were lined by rather tall regenerative epithelium. Red blood cell casts were common in the distal tubules. Some widely dilated tubules filled with hyaline casts also were present. The lumens of several proximal tubules were filled with polymorphonuclear neutrophiles, and some of the hyaline casts previously noted contained scattered polymorphonuclear leukocytes and tubular remnants embedded in them. The glomeruli were severely diseased; all showed crescents or some adhesions between peripheral capillary loops and Bowman's capsule; all were hypercellular, primarily because of a marked increase of intracapillary polymorphonuclear neutrophilic leukocytes. As many as 70 to 80 polymorphonuclear neutrophiles and 2 to 3 eosinophiles were present in each glomerulus. The leukocytes appeared to occlude the capillary loops almost completely. The crescents contained several polymorphonuclear leukocytes. Periglomerular fibrous tissue was marked and contained a few polymorphonuclear leukocytes and small collections of chronic inflammatory cells.

Two days after biopsy the patient suffered cardiac standstill, as he was being prepared for dialysis. Cardiac massage restored activity, but the patient died several hours later. An autopsy revealed a flabby heart weighing 500 g and large, swollen kidneys weighing together 810 g. The cortical surfaces were smooth, pale gray-white, and showed no petechial hemorrhages. Microscopically the kidneys showed severe "subacute" glomerulonephritis.

Case 8. A 39 year old white male (Col) noted swelling of feet and eyes, oliguria and dark brown urine 2 weeks after a sore throat. Edema, hypertension, hematuria and proteinuria were noted by his physician, who hospitalized the patient for 11 days. However, shortly after discharge, hypertension increased and he was admitted to the VA Research Hospital. On admission, edema was not present but the blood pressure was 166/118. Aside from a chronic, moderately severe dermatitis of unkown origin that mainly involved the hands and feet, the remainder of the physical examination was normal. The urine contained 1 to 2+ protein and 10 to 25 red blood cells per high-power field but only occasional leukocytes and no casts. The BUN was 22 mg per 100 ml, the PSP excretion was 21 per cent in 15 minutes and on a concentration test the maximal urine specific gravity was 1.018. Throat cultures were not obtained, but the serum ASO titer increased from 166 to 200 U and eventually decreased to 83 U. Type 12 antibodies were demonstrated in the serum 3 months after onset but had disappeared 6 months later. Serum complement remained in the normal range. Hypertension disappeared 2 months after onset as did the nitrogen retention, although the urea clearance was only 51 per cent of normal at this time. Prednisone therapy (40 mg per day) was instituted 5 months after onset, primarily for the skin rash which had flared up and become vesicular. The rash gradually improved during the 2 months of treatment. The urea clearance rapidly returned to normal during the prednisone therapy although proteinuria did not change. Microscopic hematuria had disappeared prior to prednisone therapy. Proteinuria disappeared 26 months after onset. Subsequent urinalyses and 3 Addis counts over the next 18 months were entirely normal.

The first renal biopsy (Figure 51) was obtained 66 days after onset at a time when the urine had 1+ protein and while there was still mild hypertension. One of 12 glomeruli was hyalinized. All the remaining glomeruli exhibited 1 or more lobules with thickened hypercellular lobular stalks, but some lobules were normal and lobular stalk hypercellularity was not a prominent finding; 2 to 3 leukocytes were present in each glomerulus. Three glomeruli had scars, 4 had crescents, and 7 exhibited adhesions. Moderately severe recent as well as old interstitial fibrosis disrupted much of the cortical architecture. Many red blood cell casts were present in the tubules. An arcuate artery exhibited slight subintimal fibrosis. The arterioles were normal. The second biopsy (Figure 52) was obtained 197 days after onset. A trace of protein was still present in the urine, but renal function had returned to normal. This biopsy also contained 12 glomeruli and was very similar to the first, except that the interstitial fibrosis was all old and only a few casts were noted in the tubules. A third biopsy (Figures 56, 57) was obtained 51 months after onset and 18 months after disappearance of proteinuria. This biopsy contained 9 glomeruli, 3 of which were hyalinized. The unhyalinized glomeruli were all slightly diseased, showing in most instances slight lobular stalk thickening and hypercellularity (Figure 57); 2 were almost normal (Figure 56). Crescents were not observed and only two adhesions were noted. Considerably less interstitial fibrosis was present. Only a few hyaline casts were seen in the tubules. No arteries were present, but moderately severe arteriolar changes were seen despite the fact that the patient had been normotensive for almost 50 months and that all arterioles in the two previous biopsies had been normal.

Case 9. A 22 year old white male (Swi) was admitted to a hospital elsewhere, because of a sore throat, fever of 100° to 102° F and a history of a recent weight loss of 10 pounds. On examination the tonsils were red and covered with gray exudate. The right cervical lymph nodes were enlarged. The thyroid was diffusely

enlarged and a fine tremor of the hands was noted. The blood pressure was 140/60; the white blood cell count was 11,200 per mm^3. A throat culture yielded group B hemolytic streptococci. He was treated with penicillin for 10 days. The admission urine contained no protein but did have 5 to 10 red blood cells per high-power field. Twenty-four days after onset of the first tonsillitis attack he again developed a sore throat. The physical examination was as before. The ASO titer was 500 U, subsequently decreasing to 100. Antibodies against Type 12 streptococci, but not against Types 6 and 30, were demonstrated in the serum. The serum complement, first measured 1 month after onset of the urinary abnormalities, was within the normal range for 9 months. The urine contained a trace to 1+ protein, 15 red blood cells per high-power field, and occasional hyaline and granular casts. The BUN was 12.5 mg per 100 ml, the urea clearance 88 ml per minute and the PSP excretion 35 per cent in 15 minutes. The urine continued to contain traces of protein, occasional erythrocytes and casts for 2.5 months. Subsequent urines up to 10 months after onset were normal. A thyroidectomy was performed at this time with no recurrence of the urinary abnormalities.

The first renal biopsy (Figure 67) was obtained 32 days, and the second 79 days, after onset. Traces of protein and occasional red blood cells and casts were present in the urine at the time of both biopsies. The histologic findings in each were similar. The glomeruli were normal. However, several foci of interstitial fibrosis were present, which, in the second biopsy, contained some chronic inflammatory cells. The walls of some arterioles in the largest area of fibrosis were thickened, but elsewhere the vessels were normal.

Case 10. A 20 year old white male (Shi) noted brown urine approximately 1 week after an upper respiratory infection. Shortly thereafter petechiae developed. Four years before, while hospitalized for a brain concussion, he had been told that his urine contained a few erythrocytes. This finding was not investigated further, and, so far as the patient knew, urines examined on admission to the Army 1 year later were normal. On admission to the hospital, his blood pressure was 130/80. The physical examination was normal, except for a moderate number of petechiae. Group A hemolytic streptococci were found on throat culture. The ASO titer, obtained 4 months after the infection, was 166 U. Type 6, 12, 30 or Red Lake antibodies were not found in the serum at this time. The urine contained 1+ protein, many erythrocytes and a few leukocytes. Casts were not seen. The BUN was 17 mg per 100 ml. The urea clearance varied between 95 and 139 per cent of normal. PSP excretion was normal, and after 2 months, casual urine specific gravities as high as 1.030 were recorded. The urine gradually improved and became normal 4 months after onset.

A renal biopsy, obtained 4 months after onset, and 2 days after the urine became normal, contained 17 glomeruli (Figures 65, 66). The cortical architecture was largely intact, although a few scattered tiny perglomerular foci of interstitial fibrosis composed of mature collagenous tissue were noted. These fibrotic foci were free of inflammatory cells but contained atrophic tubules lined with low cuboidal epithelium. One glomerulus was hyalinized; another contained a scarred fibrotic lobule adherent to an area of thickening in Bowman's capsule (healed crescent?) (Figure 66). Several others contained a slight amount of PAS-positive, aniline blue-positive material in some of the lobular stalks, but no focal hypercellularity (Figure 65). Sections of a large muscular artery and numerous arterioles showed no disease. Only a rare cast in some tubules, in the tiny areas of interstitial fibrosis previously mentioned, was present.

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ABBREVIATIONS FOR ALL FIGURES

BUN = blood urea nitrogen in mg per 100 ml; PSP = per cent urinary excretion of phenolsulphonphthalein in 15 minutes after the intravenous injection of 6 mg of dye. Hematuria: numbers indicate the number of erythrocytes per high-power field in a centrifuged fresh urine specimen. H & E = hematoxylin and eosin stain; PAS = periodic acid-Schiff reaction with hematoxylin counterstain; CT = Heidenhain's connective tissue stain.

FIG. 1. SAN, FIRST BIOPSY. This glomerulus is from a 24 year old man who developed clinical acute glomerulonephritis 33 days before biopsy. At the time of biopsy he had 1+ proteinuria, microscopic hematuria; BUN was 34 and blood pressure 156/90. Note the marked hypercellularity. Only a few capillary loops are widely patent. Most are obliterated by endothelial cells with the large vesicular nuclei characteristic of the early stages of acute proliferative glomerulonephritis. An endothelial cell in mitosis is indicated by the arrow. Nine polymorphonuclear leukocytes were present in an average glomerulus from this patient but are not well illustrated at this magnification. The basement membranes are clearly delineated around the edges of the glomerular lobules and are not thickened. The epithelial cells are also normal, as is Bowman's capsule (2μ, PAS, 510×).

FIG. 2. SAN, FIRST BIOPSY. A high-power view of another mitotic figure in the glomerulus shown in Figure 1. This mitosis was in another section about 4 μ deeper in the block. Note the clear attachment of the mitotic cell to the basement membrane. Bowman's capsule is at the top of the figure. A patent capillary is present beneath the capsule. The remaining capillary loops are filled with endothelial cells, the cytoplasm of which contains poorly resolved fibrillar material which is Schiff-positive. These are the hyaline fibers described by earlier authors and do not seem to be the result of fraying of the basement membrane; they are better illustrated in a color photograph of another glomerulus from this patient shown in Figure 12. (2μ, PAS, 1,940×).

FIG. 3. SER, FIRST BIOPSY. A representative glomerulus from a 40 year old man with moderately severe histologic acute proliferative glomerulonephritis. The biopsy was obtained 30 days after onset of the clinical disease, at a time when his urine contained 2+ protein and many erythrocytes; BUN was 17 and blood pressure 136/80. Note that many more capillary loops are patent than in San (Figures 1, 2, 12) but that some lobules are filled with endothelial cells with large vesicular nuclei. Some capillary loops also contain polymorphonuclear neutrophiles. Some variation in the degree of cellularity from lobule to lobule in the glomerulus is noted. The lobule at the arrow is most severely involved, showing a marked increase in the number of endothelial cells. This lobule is illustrated at higher power in Figure 4 (2μ, PAS, 430×).

FIG. 4. SER, FIRST BIOPSY. A high-power view of a part of the lobule at the arrow in Figure 3. Note the large vesicular nuclei of the endothelial cells (1,430×).

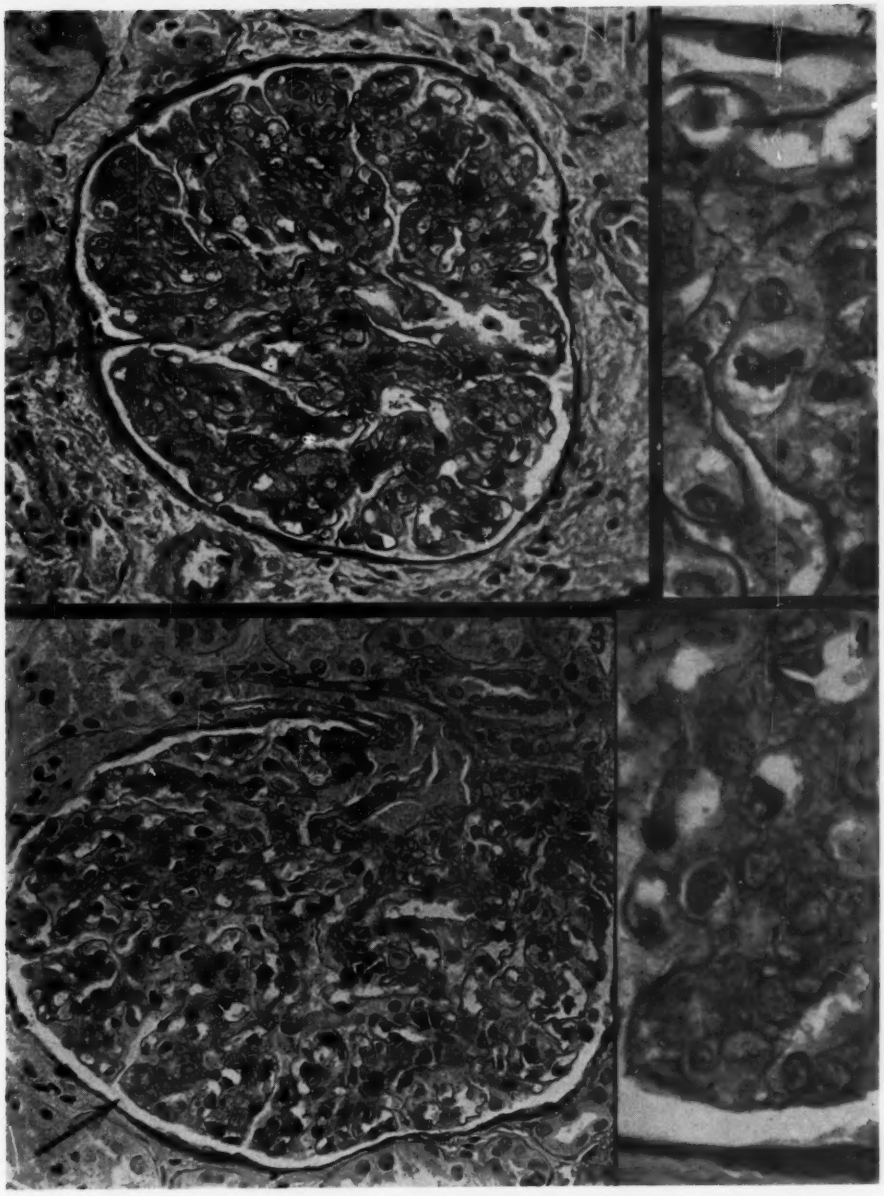

FIG. 5. BYR, FIRST BIOPSY. A low-power view of the first biopsy from a 17 year old man who had developed clinical acute glomerulonephritis 14 days previously. His urine contained 2+ protein and 20 to 40 erythrocytes; BUN was 17 and blood pressure 124/80 at time of biopsy. The cortical architecture is intact; 4 glomeruli and part of another are visible. All are hypercellular, but the two closest to the center show marked hypercellularity in contrast to the others which are only moderately so. Similar variations in degree of cellularity were common in other patients with acute proliferative glomerulonephritis in this series (2μ, H & E, 214 $\times$).

FIG. 6. BYR, FIRST BIOPSY. This glomerulus is from the biopsy of Figure 5; PAS-methenamine silver stain was used in order to accentuate the basement membranes, which stain black with this procedure. This glomerulus illustrates the variation in the degree of cellularity between different parts of the same glomerulus that occurs in acute diffuse proliferative glomerulonephritis. The lobules indicated by the arrows are almost filled with endothelial cells, while the other lobules show much less marked involvement. The cytoplasm of the epithelial cells over the lobule at the lower left contains hyaline droplets. The afferent and efferent arterioles are normal (2μ, PAS-methenamine with hematoxylin counterstain, 450 $\times$).

FIG. 7. BYR, FIRST BIOPSY. A high-power view of the lobule at the left-hand arrow in Figure 6, photographed in an adjacent serial section. Endothelial cells fill the capillary loops. Silver-positive fibrils are scattered among the cells. These hyaline fibers have the same staining characteristics as basement membrane (2μ, PAS-methenamine with hematoxylin counterstain, 900 $\times$).

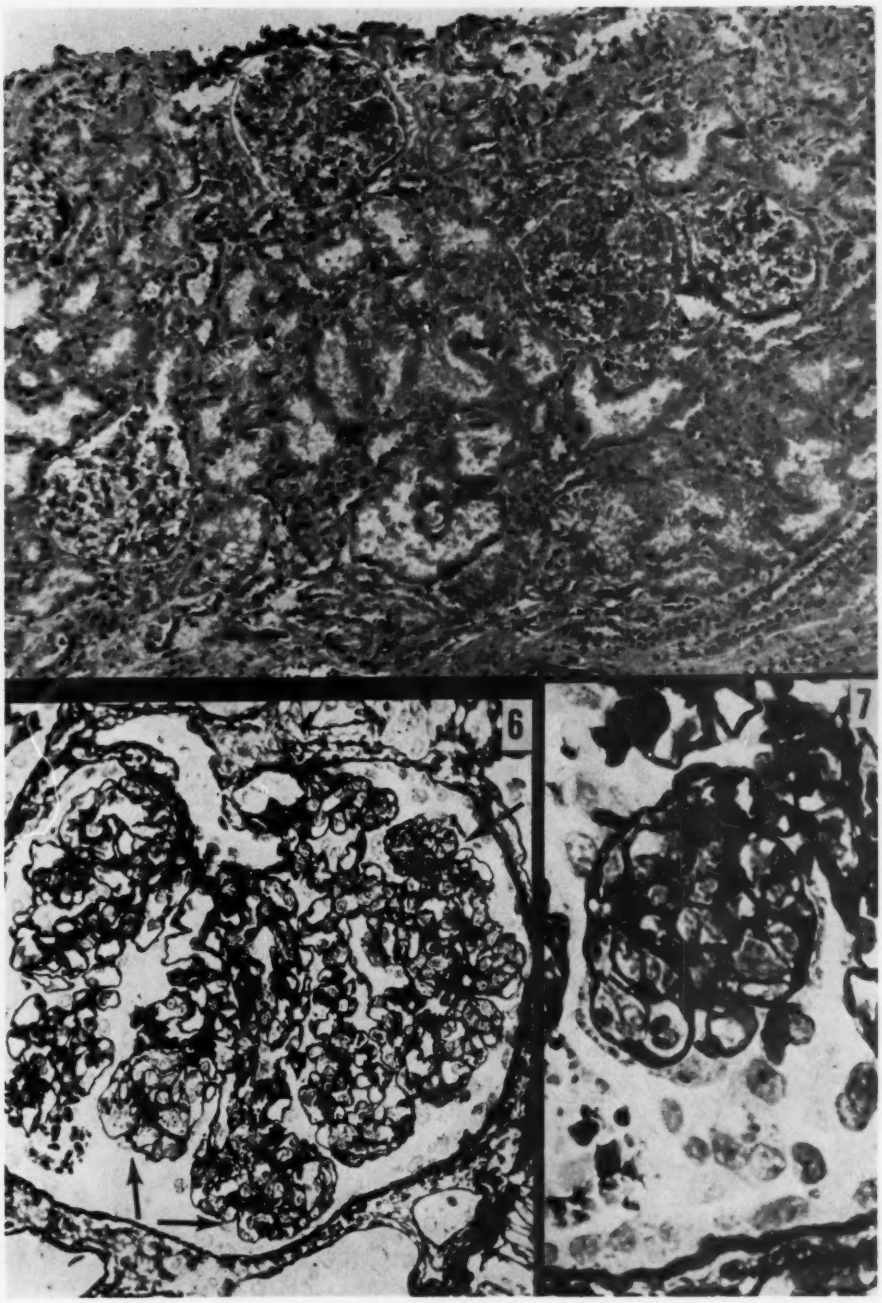
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FIG. 8. BYR, FIRST BIOPSY. Another glomerulus from the biopsy of Figure 5. It contains a crescent, presumably of recent origin (14 days or less). An adjacent section of the crescent showed a mitotic figure. An endothelial cell in mitosis is present at the lower right center. The cell is an anaphase and is illustrated in the insert at a magnification of 2,000× (2μ, PAS, H & E, 800×).

FIG. 9. MOL, FIRST BIOPSY. A representative glomerulus from a 20 year old man; biopsy obtained 22 days after onset of clinical acute glomerulonephritis. At time of biopsy this patient had 2+ protein and many erythrocytes in his urine; BUN was 19, blood pressure 135/62. All glomeruli are hypercellular but some lobules, such as the one at 2 o'clock, are much more involved than others. Note the mitotic figure in tubule cell in the lower left corner. The interstitial tissue is slightly edematous (2μ, H & E, 438×).

FIG. 10. EIK. A representative glomerulus from a 48 year old white male who was biopsied 24 days after onset of clinical acute glomerulonephritis. At biopsy he had 1+ protein and occasional erythrocytes in the urine; BUN was 19, blood pressure 126/90. The glomerulus is mildly hypercellular. An increased number of endothelial cells with rather large vesicular nuclei are noted, particularly near the central portions of the lobules. Most of the capillary loops are rather widely patent. Some contain polymorphonuclear neutrophiles; about 4 were present in this section (2μ, H & E, 328×).

FIG. 11. McC. Many glomeruli in the biopsy obtained 20 days after onset from this 59 year old white man were hyalinized due to arteriolar nephrosclerosis. He had clinical evidence of rather widespread arteriosclerosis. At time of biopsy his urine showed a trace of protein and rare erythrocytes; BUN was 17 and blood pressure 180/90. A representative unhyalinized glomerulus shows a typical, mild, acute proliferative glomerulonephritis. Note the similarity of this glomerulus to that in Figure 10. Many capillary loops are patent. Increased numbers of cells with rather large vesicular nuclei are present near the central portions of many glomerular lobules. Polymorphonuclear neutrophiles are visible in some capillary loops. The granular material in Bowman's space on the lower left is not so-called protein precipitate but is proximal tubular cytoplasm and brush border material that has been compressed in Bowman's space during the trauma of biopsy (2μ, H & E, 450×).

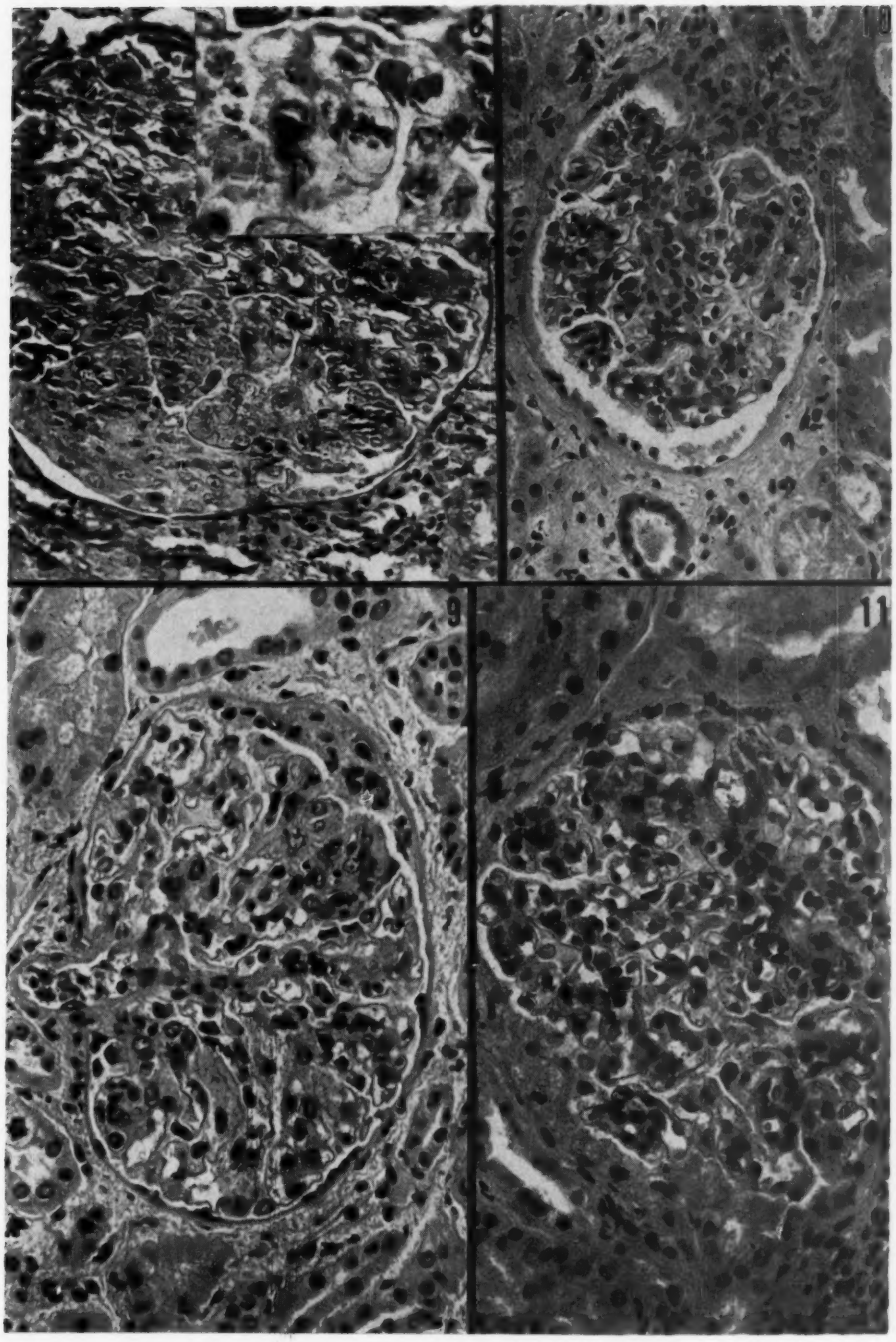
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FIG. 12. SAN, FIRST BIOPSY. A high-power view of portions of 3 lobules of a glomerulus from the same patient shown in Figures 1 and 2. Note that the lobules are filled with endothelial cells that are attached to the basement membranes and have rather large vesicular red nuclei. The cytoplasm of these cells contains fine blue hyaline fibers which are well illustrated at the upper arrow. Dense compact chromatin typical of normal glomerular endothelial cells is illustrated by the two nuclei in the capillary at the lower arrow. Reference to Figures 1 and 2 shows that few normal nuclei were present in the glomeruli of this patient. A portion of the normal afferent arteriole to this glomerulus is present in the lower left-hand corner (2μ, CT, 1,000 $\times$).

FIG. 13. LAR. This glomerulus is from a 19 year old male who developed clinical acute glomerulonephritis 79 days before biopsy. At the time of biopsy his urine contained no protein but did have occasional erythrocytes; BUN was 11, blood pressure 145/90. This glomerulus is representative and shows a striking increase in the number of nuclei within the lobular stalks along with aniline blue-positive material, which on close examination has a fibrillar pattern particularly in the lobule at 11 o'clock. Note that the basement membranes and epithelial cells are normal as is the cortical architecture (2μ, CT, 847 $\times$).

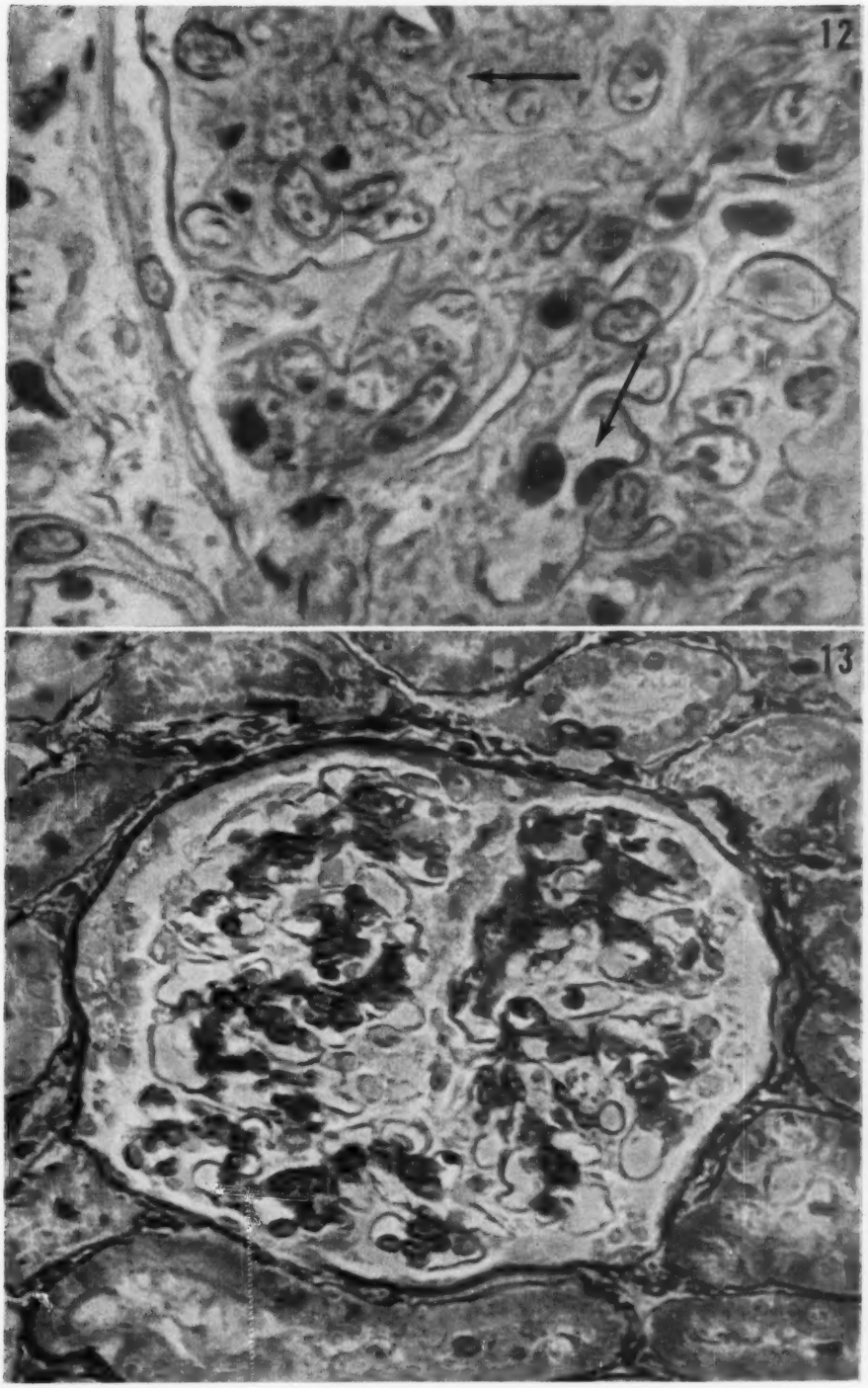
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FIG. 14. SER, SECOND BIOPSY. From the same patient shown in Figures 3 and 4, obtained 80 days after onset of disease. At this time his urine contained a trace of protein and occasional erythrocytes; BUN was 20, blood pressure 124/80. This low-power view of the middle two-thirds of the biopsy shows the cortical architecture to be intact. Each glomerulus is diseased and shows thickened lobular stalks. The glomerular changes are illustrated in detail in Figures 15 and 16 (2μ, PAS, 65×).

FIG. 15. SER, SECOND BIOPSY. A higher-power view of a glomerulus from the biopsy of Figure 14. Note the increased number of nuclei and PAS-positive material in each lobular stalk. The degree of involvement varies from lobule to lobule. Those at 3, 4, and 6 o'clock are thicker and contain more cells than the others. The basement membranes are of normal thickness and most of the capillary loops are widely patent. Compare the appearance of this glomerulus with that of the glomerulus from the first biopsy of this patient, shown in Figure 3. The hypercellularity is much more diffuse in the earlier biopsy, and the glomeruli contain more inflammatory cells and fewer patent capillary loops than do those in the second biopsy (2μ, PAS, 556×).

FIG. 16. SER, SECOND BIOPSY. Another glomerulus from the biopsy of Figure 14 to illustrate that some glomeruli in the subsiding stages of a proliferative glomerulonephritis may have much less involvement than others. The lobular stalks at the arrows are slightly thickened and hypercellular but are less diseased than the lobules of the glomerulus shown in Figure 15 (2μ, PAS-methenamine with hematoxylin counterstain, 450×).

FIG. 17. SER, THIRD BIOPSY. Obtained 76 days after the second biopsy shown in Figure 14, and 156 days after onset of the clinical disease. At the time of third biopsy the urine had been protein-free for 66 days; BUN was 13, blood pressure 138/80. Note that the lobular stalks are still hypercellular and that the general appearance of this glomerulus is similar to that of glomeruli in the second biopsy. The nuclei of the lobular stalk cells appear smaller and less vesicular than during the acute phase. Many of the nuclei also now contain prominent nucleoli. The basement membranes and the arteriole at the hilus are normal (2μ, H & E, 500×).

FIG. 18. SER, FOURTH BIOPSY. A micrograph of one of the two glomeruli obtained 775 days after onset of clinical disease and 685 days after the urine became protein-free. The lobular stalks at the arrows are slightly thicker and more cellular than normal. The other lobules are normal, as is the arteriole. The other glomerulus from this biopsy was normal (2μ, CT, 660×).

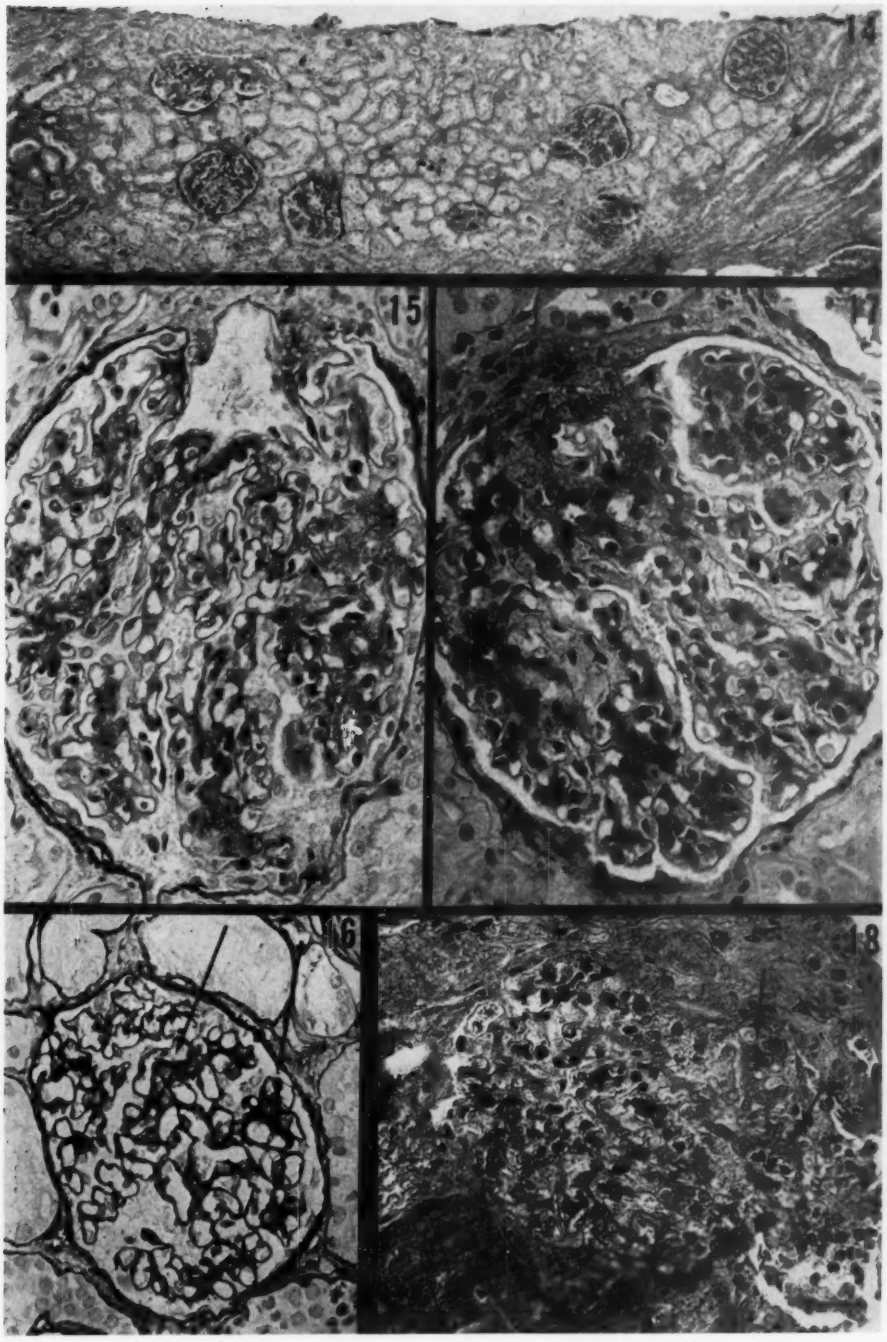
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FIG. 19. BYR, SECOND BIOPSY. Obtained 115 days after onset and 101 days after the first biopsy of Figures 5–8. At the time of second biopsy his urine had 2+ protein and 20 to 30 erythrocytes; BUN was 14, blood pressure 120/80. This is a representative glomerulus which shows small foci of lobular stalk hypercellularity. The basement membranes and tubules are normal (2μ, H & E, 356×).

FIG. 20. BYR, SECOND BIOPSY. This is another glomerulus from the biopsy of Figure 19. It illustrates abundant silver-positive material in the lobular stalks. The endothelial nuclei in this area are obscured by the silver desposits (2μ, PAS-methenamine, 356×).

FIG. 21. SAN, SECOND BIOPSY. Obtained 73 days after onset and 40 days after the first biopsy, which is shown in Figures 1, 2 and 12. At the time of second biopsy the patient's urine showed a trace of protein and occasional red blood cells; BUN was 12, blood pressure 110/52. This is a representative glomerulus and exhibits hypercellular and thickened lobular stalks similar to those in Figures 15, 17 and 19 from other patients with subsiding acute proliferative glomerulonephritis. The lobules at 1 and 3 o'clock show slightly more severe disease than the other lobules in the glomerulus (2μ, H & E, 512×).

FIG. 22. SAN, SECOND BIOPSY. This high-power view illustrates a focus of marked lobular stalk involvement from the same biopsy of Figure 21. Note that basement membranes are normal around the periphery of the loops. The nuclei in the center of the stalk are located in cytoplasm and no discrete cell membranes are present. The cytoplasm contains poorly resolved hyaline fibers that are blue with this stain and are similar to those noted in Figure 13. (2μ, CT, 1,960×).

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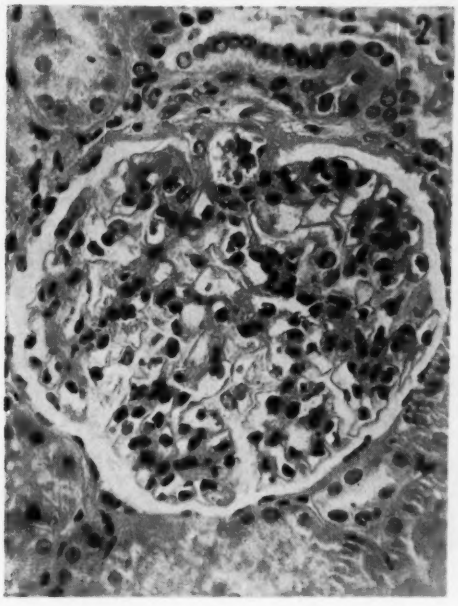
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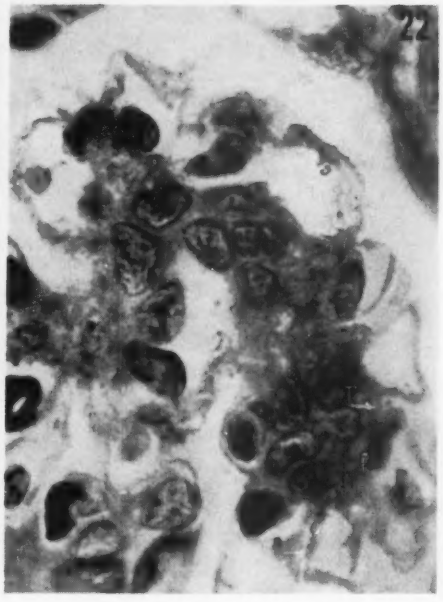
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FIG. 23. MOL, SECOND BIOPSY. Obtained 114 days after onset and 92 days after the first biopsy (Figure 9). At the time of second biopsy this patient had 1+ proteinuria and many red blood cells in his urine; blood pressure was 120/60. This low-power view shows 3 glomeruli sectioned near the midplane. The interstitium is normal. Red cell casts are present in collecting ducts near the center of the photograph. The glomeruli show lobular stalk hypercellularity of moderate severity. The glomerulus at upper left is illustrated in Figure 24 (2μ, H & E, 193×).

FIG. 24. MOL, SECOND BIOPSY. A high-power view of the glomerulus at the upper left in Figure 23; it clearly shows 3 foci of rather marked lobular stalk thickening and hypercellularity at 1, 2 and 5 o'clock (2μ, H & E, 328×).

FIG. 25. MOL, THIRD BIOPSY. Obtained 205 days after onset and 183 days after the first biopsy. At the time of this biopsy the patient's urine was normal; BUN was 8, blood pressure 125/60. This glomerulus shows some crushing artifact in that the basement membranes of the capillary loops are irregular. Note that the lobular stalk lesion is very similar to that present in the second biopsy. Bowman's capsule on the lower left is lined by cuboidal epithelium, a finding occasionally noted in normal kidneys (2μ, PAS, 850×).

FIG. 26. MOL, THIRD BIOPSY. Silver-positive hyaline fibers are indicated by the arrows in the middle of a small focus of lobular stalk hypercellularity (same biopsy as Figure 25). These fibers exhibit the staining reactions of glomerular basement membrane. They are positive to the PAS reaction, stain blue with aniline blue, and react with silver after digestion with dilute periodic acid. Collagen also shows these reactions. However, electron micrographs (Figure 27) show that these fibers lack the periodicity of collagen and we assume, therefore, that they are a product of the endothelial cells with a consistency similar to that of the basement membrane of the glomerular capillary loops. The nuclei of the cells of the lobular stalk are at n, the nuclei of the endothelial cells attached to the basement membrane are at e, and an epithelial cell is at ep (2μ, PAS-methenamine with hematoxylin counterstain, 1,850×).

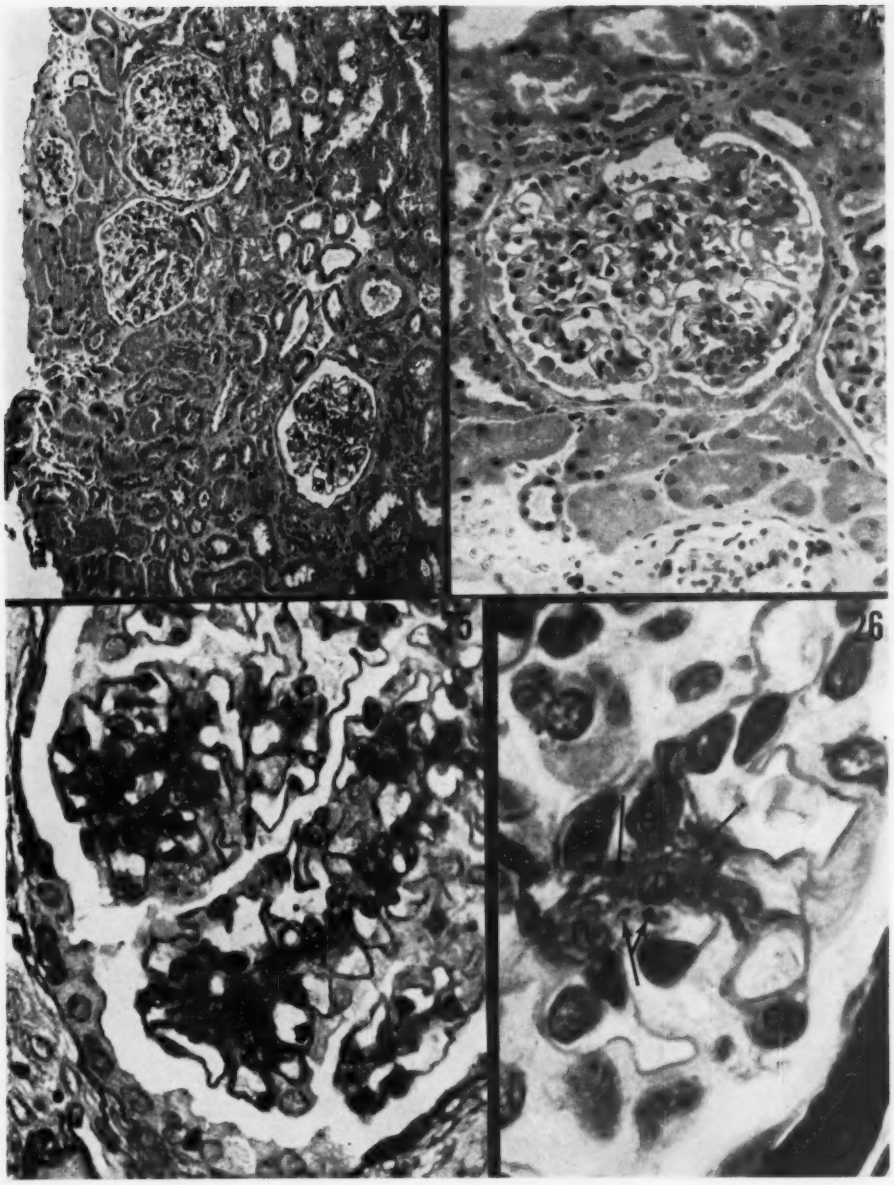
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FIG. 27. MOL, THIRD BIOPSY. Electron micrograph of a portion of a glomerulus to show one focus of lobular stalk hypercellularity under higher magnification. The basement membrane of the glomerular capillary is at bm. Normal foot processes of the epithelial cells are clearly visible over the outer surface of the capillary and a thin layer of endothelial cytoplasm is present on the lumen side. Two erythrocytes (rbc) are present inside the capillary. A hypercellular lobular stalk, which is covered by basement membrane and foot processes at the top and a thin layer of vesicular endothelium at the bottom, is present in the center of the figure. It contains 4 nuclei (n). Note that no cell membranes are present between the nuclei and that the cytoplasm of the stalk area contains numerous irregular hyaline fibers, some of which are identified by arrows. A portion of a normal endothelial cell is shown at en. No nuclei of epithelial cells are visible in the photograph (7,600×).

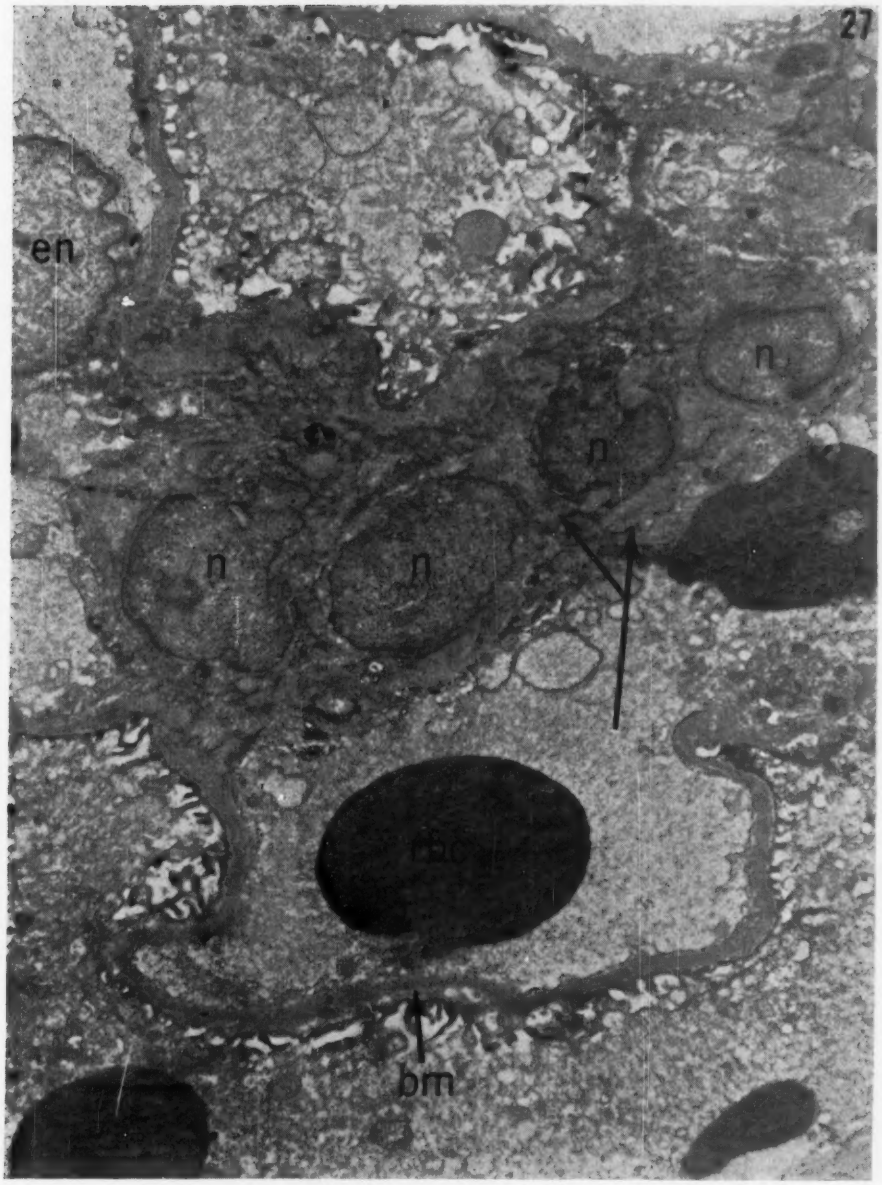
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FIG. 28. MOL, THIRD BIOPSY. Higher-power view of the lobular stalk cell containing nucleolus (nu) on the left-hand side of Figure 27. Note the 3 mitochondria (m) in the cytoplasm of the endothelial cell and a portion of normal basement (bm). The hyaline fibers at the arrows are surrounded by tubular elements of the endoplasmic reticulum. The density and structure of the fibers are similar to the basement membrane of the capillary loop at bm. Note that the hyaline fibers show none of the fibrillar pattern and periodicity of collagen. The endoplasmic reticulum appears to be connected to the nucleus in some areas, particularly near the small bit of dense osmophilic material at the top of the figure (27,750×).

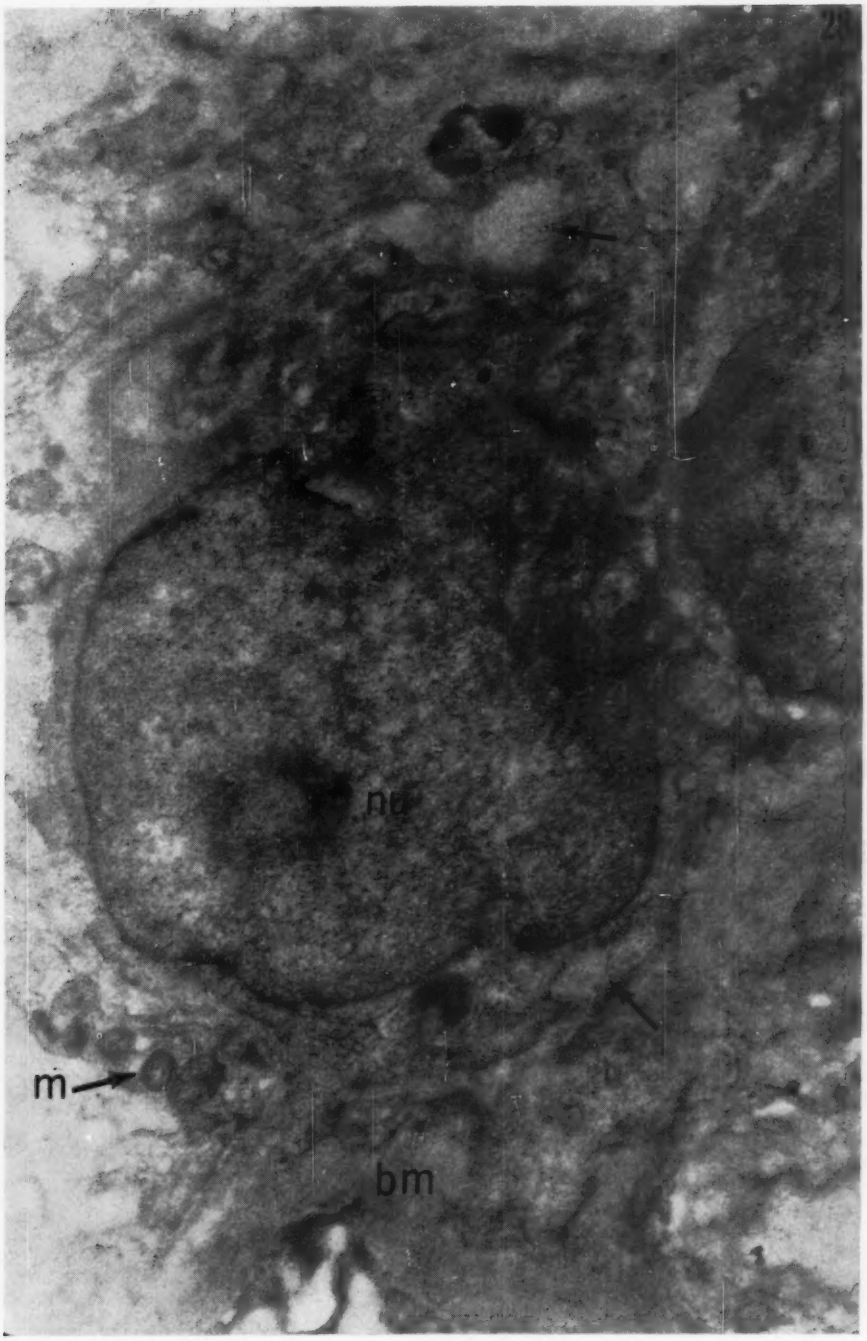
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FIG. 29. MAR. Obtained from a 57 year old white male 30 days after onset of acute post-streptococcal glomerulonephritis and illustrates a thrombus in a glomerular capillary loop. Note that the basement membrane of the capillary containing the thrombus is thickened but that the others in the section are normal. At the time of biopsy the patient's urine contained 2+ protein and 40 to 50 erythrocytes; BUN was 28, blood pressure 148/80. All lobules contain increased numbers of intracapillary cells with large vesicular nuclei. The lobules at 8 to 9 o'clock contain many more polymorphonuclear neutrophiles than other lobules in the glomerulus. This patient exhibits a mixed proliferative and focal exudative response (2μ, H & E, 470×).

FIG. 30. DIL. This glomerulus is one of the most severely involved of 22 in a biopsy obtained from an 18 year old white male 82 days after onset of a very mild clinical acute glomerulonephritis. At the time of biopsy his urine contained no protein and 10 erythrocytes; BUN was 12, blood pressure 130/80. The glomerulus shows 2 obvious thickened hypercellular lobular stalks but is otherwise normal (2μ, H & E, 484×).

FIG. 31. DIL. Another glomerulus from the patient of Figure 30. It is representative and normal except for 3 slight foci of lobular stalk thickening and hypercellularity near the center and at 1 and 5 o'clock (2μ, PAS, 625×).

FIG. 32. LAT. A low-power view of approximately one-quarter of the biopsy. The tissue was obtained 122 days after onset, at which time the urine contained 1+ protein and 1 to 3 erythrocytes; BUN was 25, blood pressure 135/95. The cortical architecture is intact. All glomeruli in the biopsy are diseased, showing lobular stalk thickening and hypercellularity (2μ, H & E, 139×).

FIG. 33. LAT. High-power view of a glomerulus from the same patient of Figure 32 to show the characteristic glomerular lesion of subsiding acute proliferative post-streptococcal glomerulonephritis. The extent of the lobular stalk involvement as to degree of thickening, hypercellularity and number of lobules markedly involved is greater than in any of the patients so far illustrated. This patient's nephritis failed to heal (2μ, H & E, 554×).

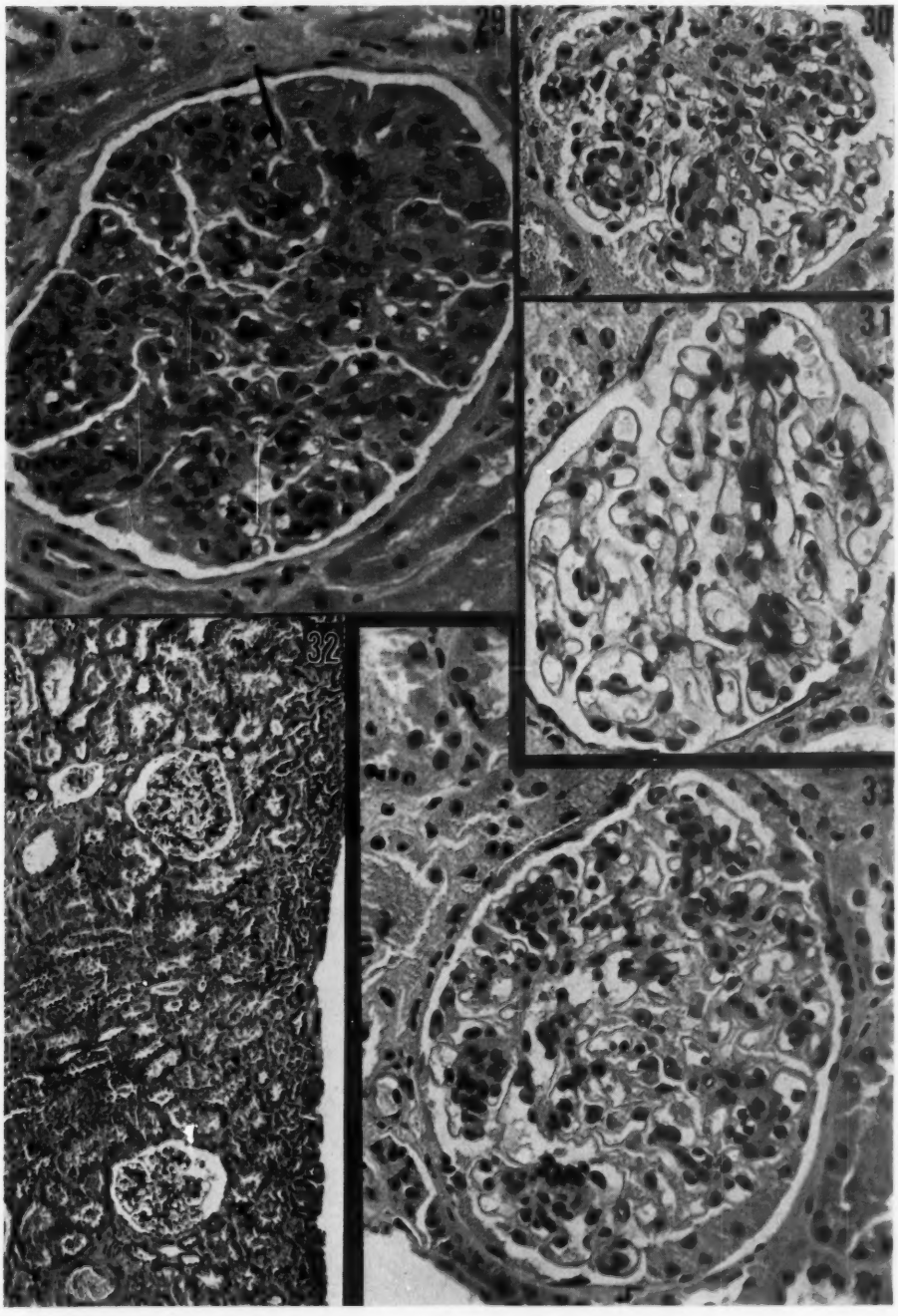
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FIG. 34. EVA. A representative glomerulus from a patient described in detail in the protocols (Case 1). At the time of biopsy his urine showed many erythrocytes but no protein; BUN was 15, blood pressure 130/80. The characteristic lobular stalk hypercellularity of an antecedent acute proliferative glomerulonephritis is clearly demonstrated (2μ, H & E, 676×).

FIG. 35. BIS. This micrograph is one of the most severely involved glomeruli found in the biopsy of a 21 year old man's kidney 146 days after onset. At the time of biopsy his urine contained 2+ protein and 20 to 50 erythrocytes; BUN was 20, blood pressure 110/80. Note adhesions at 7 and 10 o'clock and the increased intracapillary cells, primarily located in the stalks of the lobules. The black objects are erythrocytes which stain bright scarlet with azocarmine and, with a green filter, photograph deep black (2μ, CT, 495×).

FIG. 36. SCH. This micrograph includes on the left the most severely involved of the 36 glomeruli obtained from this 18 year old white male 132 days after onset of disease; seven days after this biopsy his urine became protein-free. At the time of biopsy his urine showed a trace of protein and 2 to 4 erythrocytes; BUN was 15, blood pressure 122/66. The right-hand glomerulus is not representative of the glomeruli in the biopsy, since it shows less lobular stalk hypercellularity than most of the other glomeruli. The left-hand glomerulus shows a lobular scar and adhesion (2μ, H & E, 309×).

FIG. 37. MOE. A low-power view from a biopsy taken from an 18 year old white male 170 days after onset. At the time of biopsy his urine showed a trace of protein and an occasional erythrocyte; BUN was 18, blood pressure 125/80. This representative glomerulus shows the lobular stalk involvement characteristic of the subsiding phase of post-streptococcal proliferative acute glomerulonephritis (6μ, PAS, 326×).

FIG. 38. ROO, FIRST BIOPSY. A glomerulus from a biopsy taken 47 days after onset in a 59 year old white male. At the time of biopsy his urine showed 1+ protein and rare erythrocytes; BUN was 20, blood pressure 120/70. This glomerulus is representative of mild acute proliferative glomerulonephritis. The endothelial cells which are present in slightly greater than normal numbers have large vesicular nuclei (2μ, H & E, 450×).

FIG. 39. ROO, SECOND BIOPSY. Obtained 602 days after onset and 249 days after the urine became protein-free. This glomerulus is normal, as were all others in the biopsy (2μ, PAS, 450×).

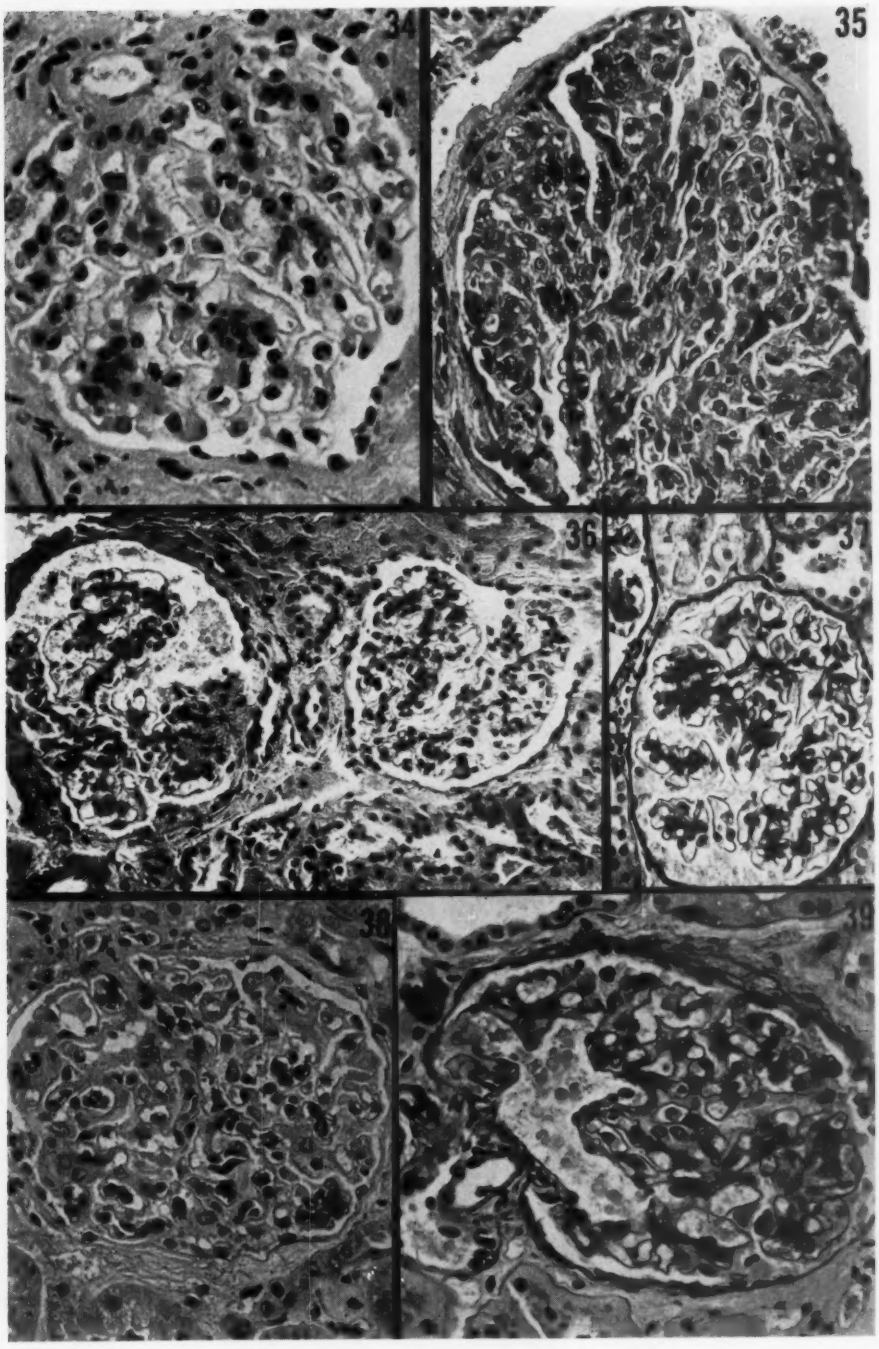
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FIG. 40. APE, FIRST BIOPSY. See Case 6 in the protocols for detailed history. This 39 year old woman had severe clinical acute glomerulonephritis. A low-power view of about one third of the biopsy illustrates the severe cortical edema shown by the entire biopsy; at this time her urine showed 3+ protein and many red cells and red cell casts; BUN was 52, blood pressure 180/100. The black casts in the center of the micrograph are red cell casts in collecting ducts (2μ, CT, 59×).

FIG. 41. APE, FIRST BIOPSY. This hypercellular glomerulus is from the same biopsy shown in Figure 40. About 20 acute inflammatory cells are present in the capillaries and some lobules show a slight increase in the number of endothelial cells. Note the cells with large vesicular nuclei at the arrow (2μ, H & E, 450×).

FIG. 42. APE, FIRST BIOPSY. Another glomerulus from the biopsy of Figure 40. A large crescent is present on the lower half of Bowman's capsule. The capillary loops are adherent to the crescent at 7 o'clock and to Bowman's capsule at 1 o'clock (6μ, CT, 281×).

FIG. 43. APE, FIRST BIOPSY. A necrotic glomerulus from another section of the biopsy of Figure 40. The glomerulus is infiltrated with numerous polymorphonuclear neutrophiles and adherent to Bowman's capsule over one-half of its circumference. The adjacent interstitial tissue is edematous and the tubules which are present are lined by flattened or regenerative epithelium. The tubule at the upper left contains a red cell cast (2μ, H & E, 332×).

FIG. 44. APE, FIRST BIOPSY. A glomerulus from another part of the biopsy of Figure 40. Many of the capillary loops have a diminished diameter because of swelling of their endothelial lining (arrow at center) while others are occluded by polymorphonuclear neutrophiles. The polymorphonuclear cells have completely occluded the lobule at the upper left. This represents severe focal exudation, and is a phenomenon that we believe is often followed by lobular scarring (see Figure 50 for the appearance of the possible end-stage of this lesion). Note that new endothelial cells are not common and that the chief cause of the loss of glomerular filtering surface in this patient is edema and the polymorphonuclear cell infiltration. The basement membranes are normal and no capillary thrombi are present (2μ, PAS-methenamine with hematoxylin counterstain, 850×).

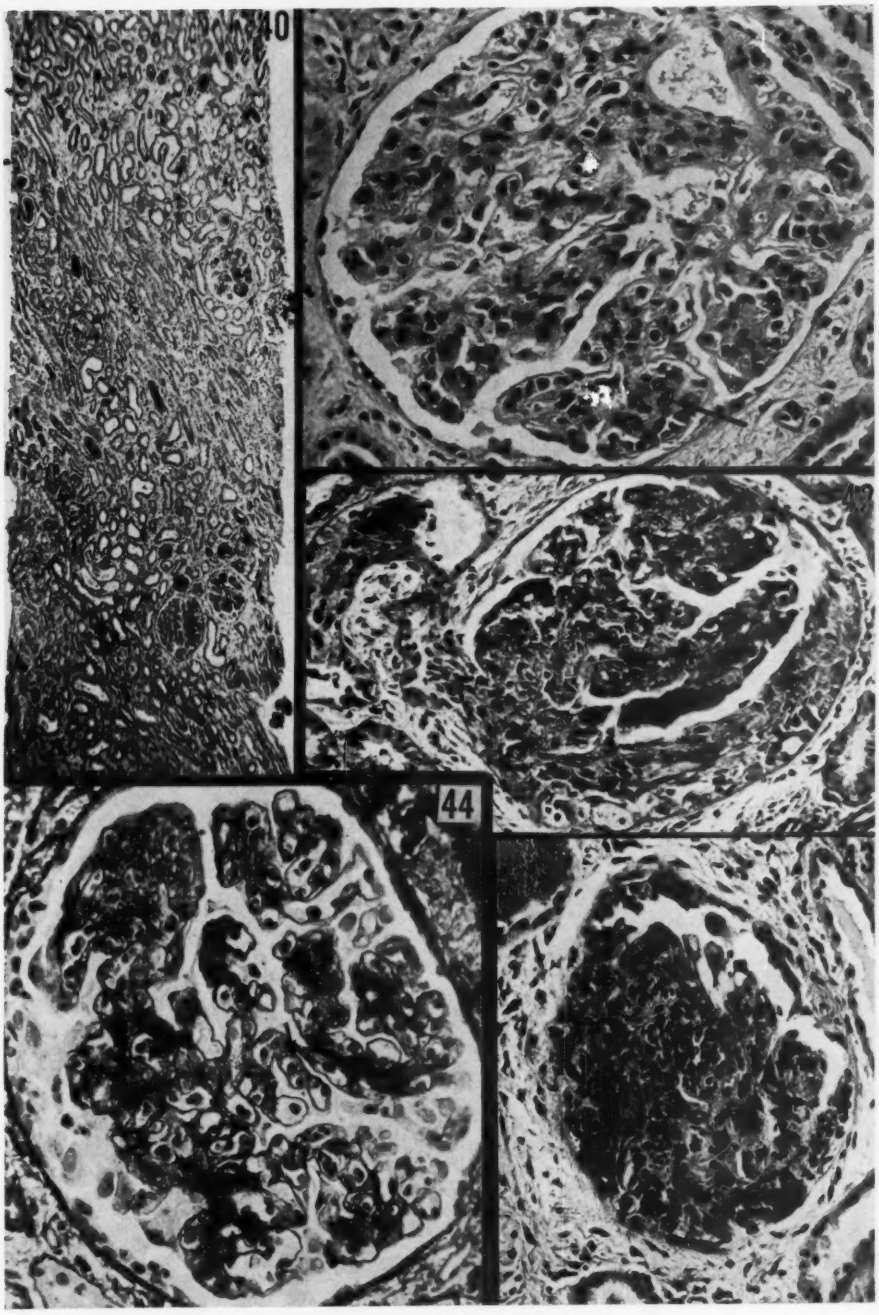
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FIG. 45. CAL. This low-power micrograph shows 2 glomeruli and the interstitial tissue from the most severe acute exudative glomerulonephritis observed in this series. This patient is described in detail in the protocols as Case 7. The biopsy was obtained 30 days after onset and 2 days prior to death. The glomeruli are markedly hypercellular and contain crescents. The interstitial tissue is edematous and contains focal collections of polymorphonuclear neutrophiles and lymphocytes. The proximal tubules are widely separated by edema. At autopsy this kidney weighed 400 g (2μ, H & E, 120×).

FIG. 46. CAL. A higher-power view of the right-hand glomerulus in Figure 45, which shows that the hypercellularity is due to numerous polymorphonuclear neutrophiles in the capillary loops and to 3 foci of epithelial cell proliferation (crescents) indicated by the arrows. This is an example of almost pure acute exudative glomerulonephritis. There is no increase in the number of endothelial cells in this glomerulus in the other glomeruli in the biopsy (2μ, H & E, 276×).

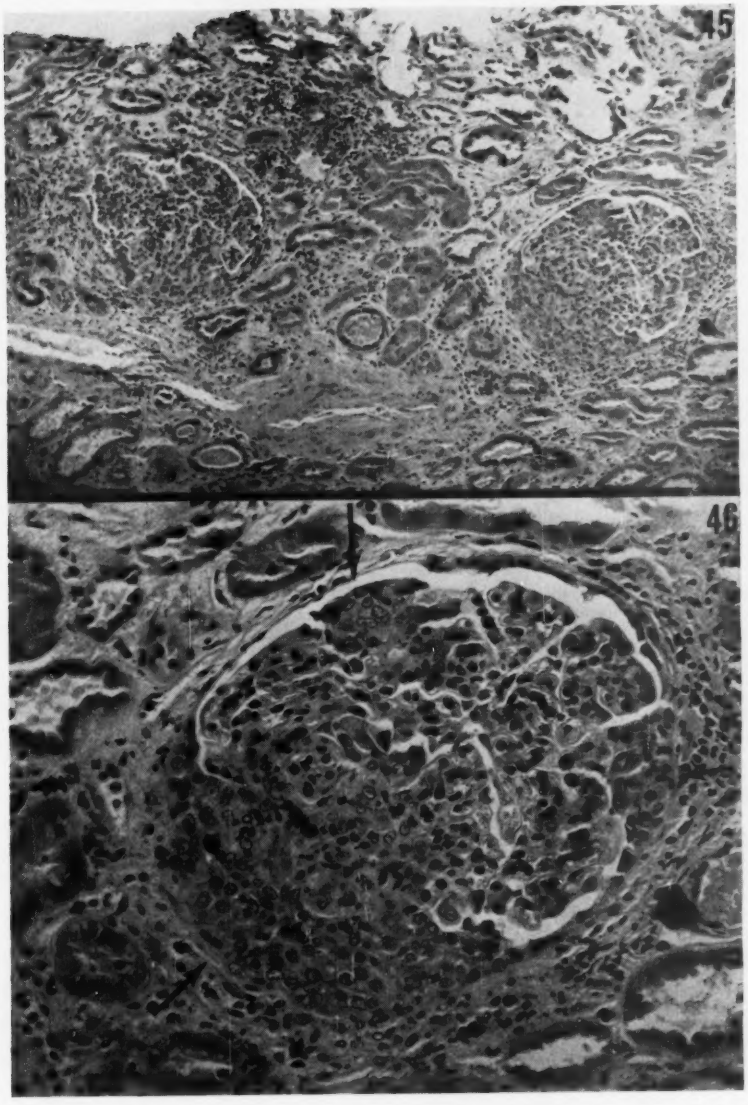
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FIG. 47. CAL. This micrograph shows a portion of a glomerulus from the same patient of Figure 46. An epithelial cell (ep) is at upper left center. The epithelial cell foot processes cover the basement membrane but, except for being less dense than normal in some areas, are not remarkable. The basement membranes (bm) are intact, and the endothelial layer covering the membrane internally is slightly swollen. Attached to the endothelium are several polymorphonuclear neutrophiles (p). Two erythrocytes (rbc) are in the lumens of the capillaries. Four nuclei (e) of mature endothelial cells are noted (7,000×).

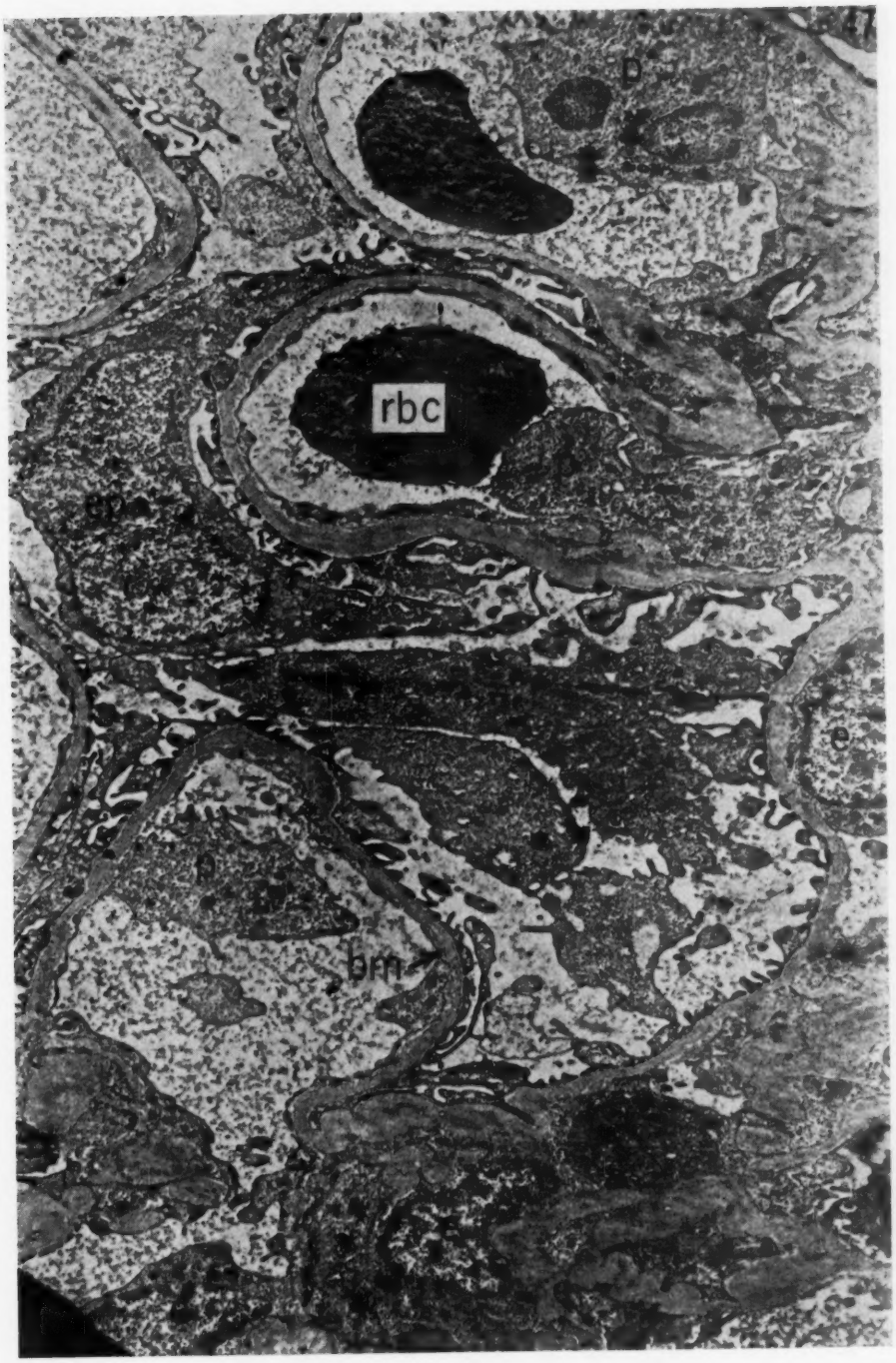
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FIG. 48. APE, SECOND BIOPSY. This patient was first discussed in Figure 40; the second biopsy was obtained 69 days after onset and 47 days after the first biopsy; at this biopsy her urine contained 2+ protein and 10 to 15 erythrocytes; BUN was 38, blood pressure 140/100. Note that the proximal tubules are widely separated by fibrous tissue and that most of the tubular epithelium is flattened and cuboidal (regenerative or atrophic type epithelium). Some normal proximal tubules are adjacent to the glomerulus on the upper right. The capillary loops of the glomeruli are widely patent (2μ, CT, 166×).

FIG. 49. APE, SECOND BIOPSY. Another glomerulus from the same patient of Figure 48. The lobular stalks are slightly thickened and hypercellular and the capillary loops are free of inflammatory cells (2μ, H & E, 436×).

FIG. 50. APE, SECOND BIOPSY. Another glomerulus from the same patient of Figure 48. Note the lobular scar and adhesion on the right (6μ, PAS, 356×).

FIG. 51. COL, FIRST BIOPSY. Glomerulus from biopsy obtained 66 days after onset in a 39 year old man (Case 8 in the protocols). At the time of this biopsy his urine contained 2+ protein and 10 to 15 red cells; BUN was 14, blood pressure 142/100. This glomerulus shows part of a healed crescent and a scarred lobule at the lower right, a relatively normal lobule at lower left and lobules with varying amounts of lobular stalk thickening and hypercellularity elsewhere in the glomerulus. The surrounding tissue (not illustrated in figure) was normal (2μ, PAS, 480×).

FIG. 52. COL, SECOND BIOPSY. Glomerulus biopsy obtained 197 days after onset from patient of Figure 51. His urine contained a trace of protein and no red cells at this time; BUN was 15, blood pressure 128/78. Most of the glomerulus is hyalinized and adherent to Bowman's capsule (2μ, CT, 520×).

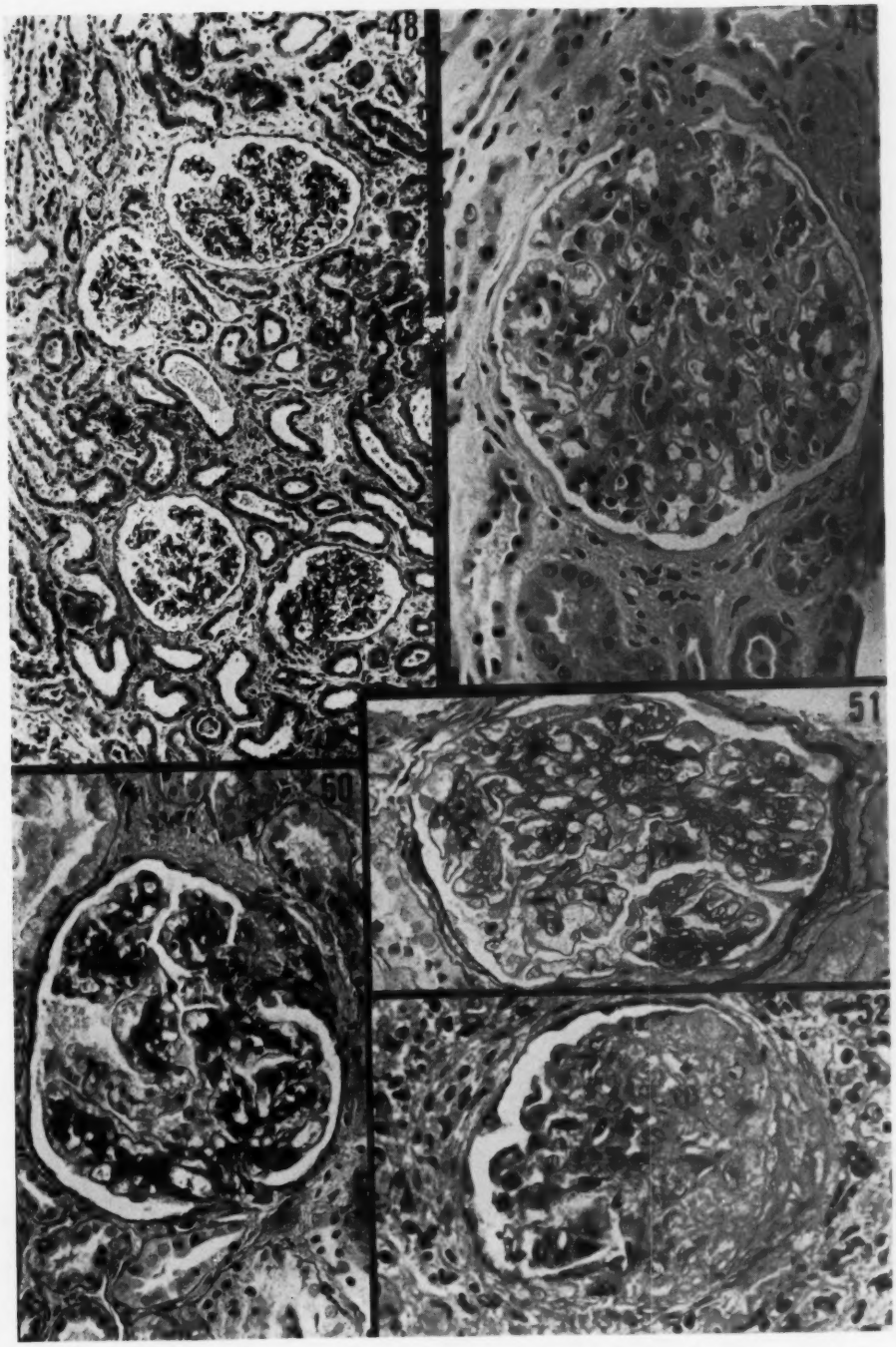
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FIG. 53. ROB. Low-power view of area of interstitial fibrosis from biopsy obtained 97 days after onset in a 20 year old white male. At this time his urine contained 1+ protein and 50 to 100 red cells; BUN was 24, blood pressure 120/80. Note that both glomeruli in the figure (arrows) show lobular scarring and partial hyalinization and that the cortex is largely replaced by dense collagenous tissue. Glomeruli from a less scarred area are shown in Figure 55 (6μ, CT, 164×).

FIG. 54. ROB. High-power view of a scarred glomerulus from general region illustrated in Figure 53. Note that almost every capillary loop is obliterated (6μ, CT, 450×).

FIG. 55. ROB. Two glomeruli from a much less scarred portion of the biopsy than that illustrated in Figures 53 and 54. The tubules are separated by an increased amount of interstitial fibrous tissue. Both glomeruli show the lobular stalk thickening and hypercellularity characteristic of the subsiding phase of proliferative glomerulonephritis. This patient presumably had a mixed initial histopathologic picture in which there was sufficient focal exudation in some glomeruli to yield the scarring shown in Figures 53 and 54, superimposed upon a primarily proliferative response (2μ, H & E, 324×).

FIG. 56. COL, THIRD BIOPSY. Normal glomerulus from patient illustrated in Figures 51 and 52. This biopsy was obtained 51 months after onset and 18 months after the proteinuria had disappeared (2μ, H & E, 280×).

FIG. 57. COL, THIRD BIOPSY. Another glomerulus from the same biopsy of Figure 57. This glomerulus shows on the left an adhesion of a glomerular capillary loop to Bowman's capsule and hypercellular thickened lobular stalks, 3, 10 and 11 o'clock (2μ, H & E, 280×).

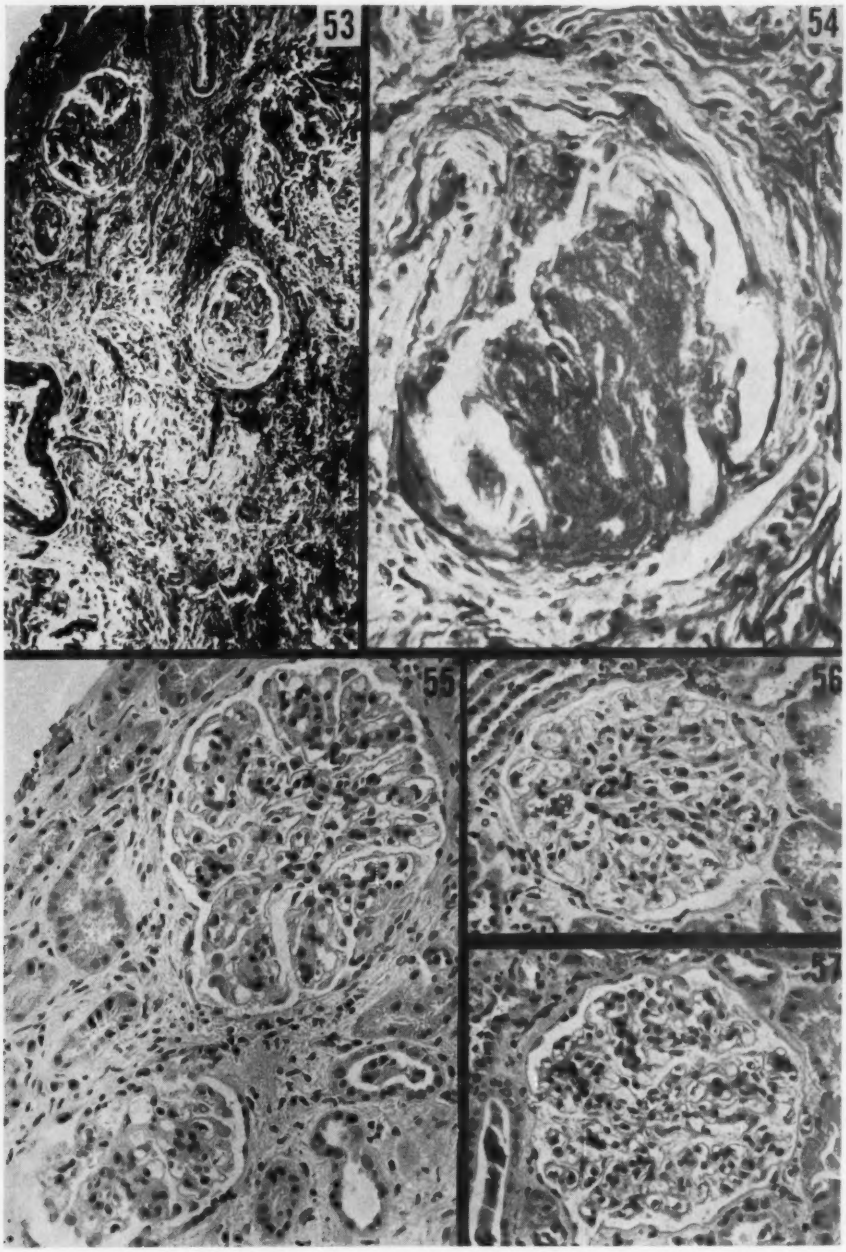
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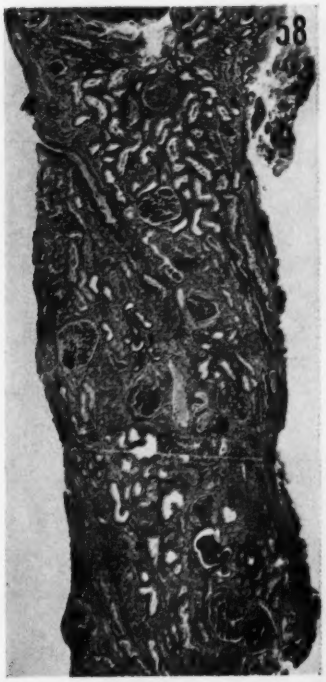
FIG. 58. VLA, FIRST BIOPSY. Low-power view of biopsy obtained 82 days after onset from a 37 year old white male. His urine contained 1+ protein and many red cells at the time of biopsy; BUN was 45, blood pressure 138/100. Note the diffuse disruption of cortical architecture by interstitial fibrous tissue. Several hyalinized and partially hyalinized glomeruli are visible (2μ, CT, 55×).

FIG. 59. VLA, FIRST BIOPSY. High-power view of glomerulus at very upper left in Figure 58. Note the lobular scar and healing crescent involving most of the lower right-hand side of the glomerulus. The remaining glomerular lobules are within normal limits. The cortex adjacent to this glomerulus is fibrotic and contains few tubules (2μ, CT, 350×).

FIG. 60. VLA, FIRST BIOPSY. High-power view of a glomerulus from the biopsy of Figure 58. The silver stain shows that most of the capillary loops are widely patent and that the basement membranes are of normal thickness. The stalks of most of the lobules of the glomerulus, particularly those at 2 and 3 o'clock, are thickened. Note the adhesion at 4 o'clock. This is filled with silver-positive collagen fibrils. The thick black line running to the lower right of the photograph is caused by a fold in the section (2μ, PAS-methenamine with hematoxylin counterstain, 554×).

FIG. 61. VLA, FIRST BIOPSY. Another glomerulus from the biopsy of Figure 58. The lobules at the arrows are thickened and hypercellular (2μ, CT, 346×).

FIG. 62. VLA, SECOND BIOPSY. Almost normal glomerulus from a biopsy obtained 162 days after onset and 80 days after the first biopsy from the same patient of Figures 58–61. His urine contained 1+ protein and occasional red cells at this time; BUN was 27, blood pressure 120/72 (2μ, H & E, 532×).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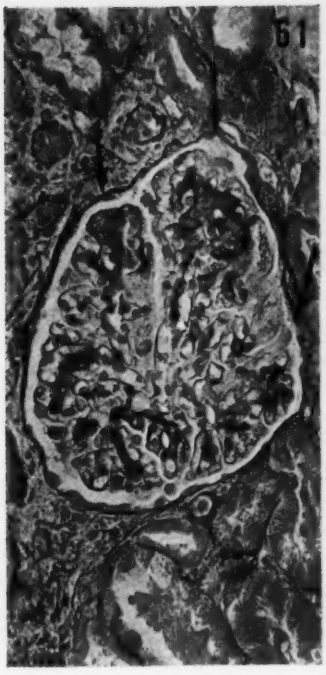
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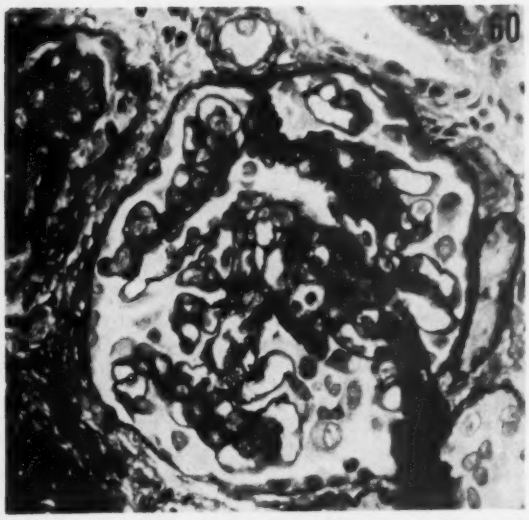
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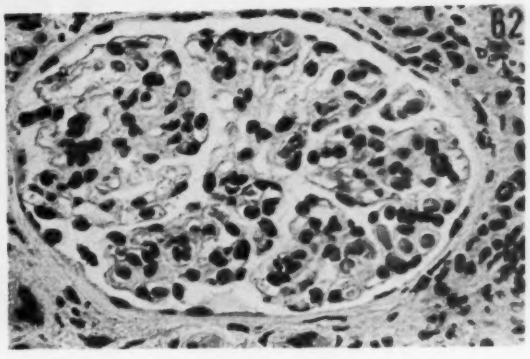
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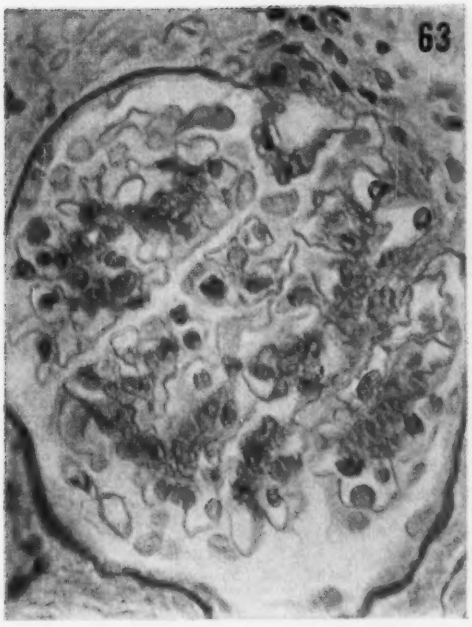
FIG. 63. TRA, FIRST BIOPSY. Glomerulus from a biopsy from a 19 year old white male 71 days after onset (Case 5 in the protocols). The urine contained 1+ protein and occasional red blood cells at this time; BUN was 13, blood pressure 135/80. Note the marked lobular stalk thickening and hypercellularity; basement membranes and arterioles at the hilus are normal (2μ, PAS, 512×).

FIG. 64. TRA, FIRST BIOPSY. Severely diseased glomerulus from biopsy of Figure 63. Note the healing crescents at the bottom and upper left. The lobular stalks show thickening and hypercellularity. The lobules adjacent to the crescents are obliterated and adherent (2μ, PAS, 840×).

FIG. 65. SHI. Normal glomerulus obtained in biopsy 120 days after onset, and 2 days after disappearance of proteinuria in a 20 year old male; Case 10 in protocols (2μ, PAS, 525×).

FIG. 66. SHI. Another glomerulus from patient of Figure 65. This biopsy showed both diseased and normal glomeruli. This is the most severely diseased glomerulus found. A healing crescent with a scarred glomerular lobule adherent to it is visible on the left. A few other glomeruli showed occasional thickened hypercellular lobules similar to that at 3 o'clock in this glomerulus (2μ, PAS, 525×).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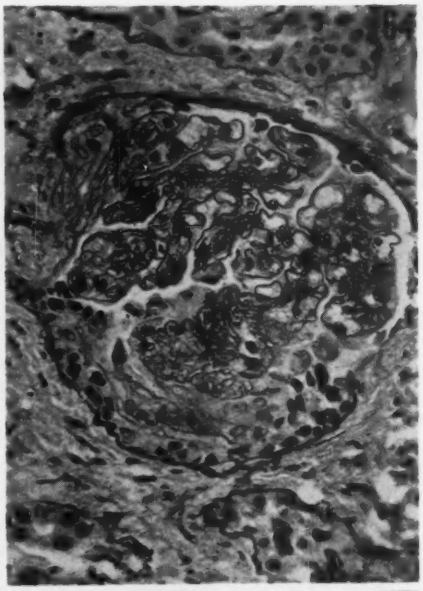
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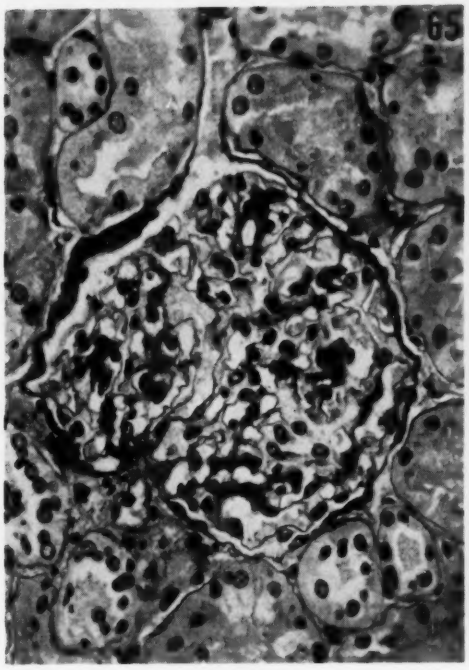
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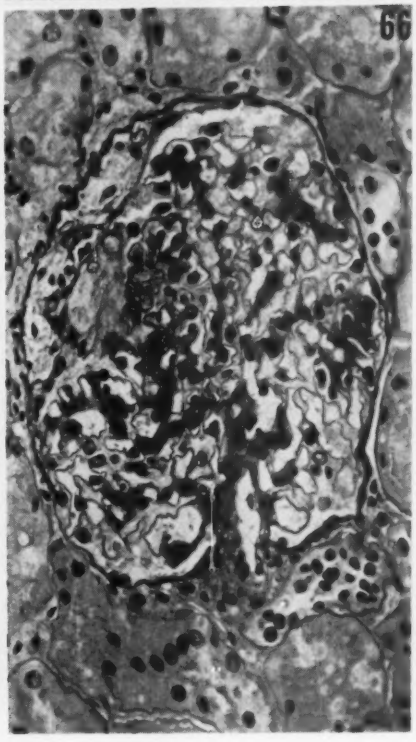
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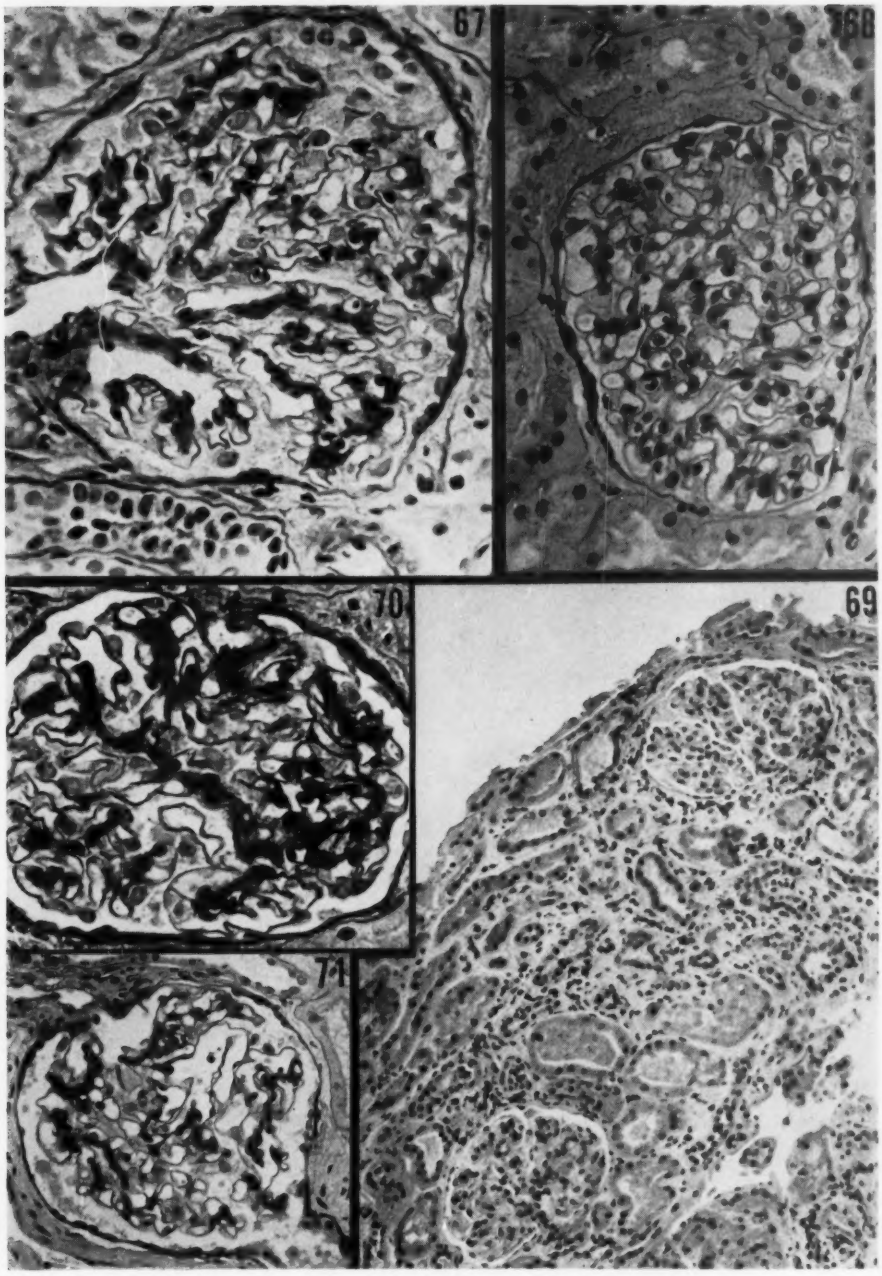
FIG. 67. SWI, FIRST BIOPSY. Normal glomerulus from first biopsy obtained from a 19 year old man 32 days after onset (Case 9 in the protocols). This patient's disease was diagnosed by laboratory findings only. At this biopsy his urine contained a trace of protein and occasional red blood cells; BUN was 12, blood pressure 140/60. A crushing artifact that commonly occurs in needle biopsies of kidney is well illustrated by this glomerulus. The basement membranes of some of the capillary loops are wrinkled, having been squashed together by the trauma of forcing the cortical tissue into the biopsy needle. This phenomenon is more common in normal than in abnormal kidneys. Swollen or fibrotic kidneys usually do not show much crushing of glomeruli or tubules except along the cut edge of the tissue (2μ, PAS, 825 $\times$).

FIG. 68. ROS. A representative normal glomerulus from a biopsy obtained 17 days after onset of disease in a 20 year old white male. His urine showed no protein and 10 red cells at this time; BUN was 15, blood pressure 120/70 (2μ, PAS, 450 $\times$).

FIG. 69. JAM, FIRST BIOPSY. Low-power view of kidney from a 26 year old Negro male biopsied 28 days after onset. His urine showed a trace of protein and a few red cells at this time; BUN was 19, blood pressure 134/90. The interstitium is fibrotic and contains an infiltrate of chronic inflammatory cells and a few polymorphonuclear neutrophiles. The glomeruli are not hypercellular and show no signs of exudation. The endothelial nuclei are all small and have dense chromatin (2μ, H & E, 234 $\times$).

FIG. 70. JAM, FIRST BIOPSY. High-power view of another glomerulus from the patient of Figure 70. Note that basement membranes are normal, the glomerulus is not hypercellular and that it is free of inflammatory cells (6μ, PAS, 510 $\times$).

FIG. 71. JAM, SECOND BIOPSY. Glomerulus from second biopsy obtained 1,013 days after onset and 985 days after first biopsy in the patient of Figures 69 and 70. At this time his urine contained 2+ protein and no erythrocytes; BUN was 14, blood pressure 150/90. This glomerulus is representative and is not abnormal (2μ, PAS, 282 $\times$).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